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DESCRIPTION

SHEAR STRESS-RESPONSIVE DNAField of the Invention

This invention relates to novel DNAs obtained by employing the subtraction method while paying attention to mRNAs which show a shear stress-dependent increase of expression in vascular endothelial cells; and proteins encoded by these DNAs. Moreover, this invention also relates to antibodies against the proteins; methods for detecting the proteins and the DNAs; and the diagnosis and treatment of various vascular diseases caused by arteriosclerosis, such as cardiac insufficiency, restenosis after PTCA (percutaneous transluminal coronary angioplasty) and hypertension, and methods for screening an agent for such treatment or diagnosis.

Background of the Invention

Conventionally, vascular endothelial cells covering the inner surfaces of blood vessels in the form of a monolayer have been considered to be a mere lining for separating the vascular tissue from blood flowing through the lumen of the blood vessel. However, as a result of the recent progress of research on the vascular endothelium, it has been found that the endothelium has a great diversity of functions which are very important for the living body. These functions include, for example, regulation of the material permeability between blood and tissue, regulation of the tension of the blood vessel, maintenance of an antithrombogenic activity, control of the proliferation of smooth muscle, repair of tissues, inflammatory reaction, and remodeling of the blood vessel. The physical force applied to the vascular wall by a flow of blood is called a shear stress, which is defined by the flow velocity of blood, the viscosity of blood, and the diameter and morphology of the blood vessel. The shear stress acts on the endothelium covering the inner surface of the vascular wall and distorts vascular endothelial cells in the direction of the blood flow. According to investigations made for the last ten years or so, it has been revealed that, similarly to chemical stimuli such as hormones and cytokines, this physical stimulus is closely associated with the

morphology of vascular endothelial cells and regulation of the above-described various functions [Cell Technology (in Japanese), 16, 950(1997)].

In industrially advanced countries including Japan, atherosclerosis is one of the major causes of death of adults. It is known that the malfunction of blood vessels caused by hypercholesteremia, hyperhomocysteinemia, diabetes mellitus and the like is closely related to the development of atherosclerosis and the aggravation of the morbid state [Molecular Cardiovascular Medicine, 49-61(1995)]. On the other hand, it is also known that arteriosclerotic lesions are not uniformly distributed over all blood vessels, but are localized in specific regions such as the outside of a bend in a branched part of a blood vessel. Since such local development is also observed in experimental animal having a genetically increased blood cholesterol level, it is considered that the incorporation of cholesterol into the vascular endothelium occurs in two stages, i.e., local changes of vascular endothelial cells and the actual incorporation of cholesterol [Arterioscler. Thromb., 14, 133-140(1994)]. The cause of such local development has scarcely been clarified. However, since incipient lesions occur frequently in places where the intensity and direction of a shear stress are not steady, i.e., places where a low shear stress is produced and the separation or stagnation of a flow or turbulence (e.g., eddies) tends to occur, hemodynamic stresses such as shear stresses are considered to be closely related to the development of atherosclerosis. At present, the molecular mechanism by which a shear stress induces arteriosclerosis locally is not clearly understood. However, genes whose expression is altered by applying a shear stress mechanically to vascular endothelial cells cultured in vitro have been searched until now. Thus, it has been found that a shear stress activates various transcription factors such as AP(activator protein)-1 and NF(nuclear factor)- κ B, and thereby causes a change of expression of genes under the control of these transcription factors. Up to now, it has been reported that the proteins encoded by genes exhibiting an alteration of expression in response to a shear stress stimulus include growth factors such as PDGF (platelet-derived growth factor) and TGF(transforming growth factor)- β ; adhesion factors such as VCAM(vascular cell adhesion molecule)-1 and ICAM(intercellular adhesion molecule)-1; tension control factors such as

ET(endothelin)-1; thrombolysis factors such as t-PA (tissue-type plasminogen activator); enzymes such as NOS (nitric oxide synthase) 3, COX (cyclooxygenase) 2 and SOD (superoxide dismutase); and the like [Molecular Medicine Today, 5, 40(1999)]. Thus, the genes responding to a shear stress in an in vitro reconstituted system are believed to include two groups of molecules having different characteristics, i.e., arteriosclerosis induction factors considered to be expressed in at least low shear stress regions of blood vessels in response to a change of shear stress, and molecules suppressing the development of arteriosclerosis in intravascular places where a high shear stress is produced constitutively. However, among the genes presumed to exhibit an alteration of expression in response to a shear stress only some genes have been specifically identified. In order to understand the cause of arteriosclerosis and develop methods for the prevention and treatment thereof, it is necessary to clarify unknown genes responding to a shear stress. In recent years, unknown genes responding to a shear stress have been searched by employing the differential display method or the like, but it involves several problems in that genes whose alteration of expression is of the order of several times cannot be easily obtained and in that the proportion of false positive clones is high [Nucleic Acids Res., 23, 4520-4523(1995)]. Consequently, the number of genes exhibiting an alteration of expression in response to a shear stress and clarified by the differential display method is not great [Proc. Natl. Acad. Sci. USA, 93, 10417-10422(1996); Proc. Natl. Acad. Sci. USA, 94, 9314-9319(1997); Biochem. Biophys. Res. Comm., 255, 347-351 (1996); Biochem. Biophys. Res. Comm., 246, 881-887(1998); US Patent 5,834,248 (1998); US Patent 5,849,578 (1998); US Patent 5,882,925 (1999)].

As described above, it is recognized that changes of the shear stress applied to vascular endothelial cells are involved in the local development of atherosclerosis, but the fact is that its molecular mechanism is scarcely understood. Nevertheless, it has been reported for long that a shear stress reduces the turnover of endothelial cells in vivo, i.e., a shear stress acts so as to suppress the cell death of the endothelium [Atherosclerosis, 17, 401-417(1973); Circ. Res., 69, 1557-1565(1991)]. Moreover, there are many reports showing that, in vitro, the apoptosis of endothelial cells induced by TNF- α stimulation, hydrogen peroxide stimulation,

growth factor depletion or the like is markedly suppressed by the application of a shear stress [J. Exp. Med., 185, 601-607(1997); FEBS Lett., 399, 71-74(1997); Arterioscler. Thromb. Vas. Biol., 17, 3588-3592(1997); Biochem. Biophys. Res. Commun., 231, 586-590(1997)]. That is, it is believed that, in branched or curved parts of arteries where a low shear stress is produced, the character of endothelial cells changes so as to induce apoptosis and this is a cause defining the locality of an incipient arteriosclerotic lesion. At present, however, little is known about genes participating in the molecular mechanism by which the application of a shear stress suppresses the apoptosis of endothelial cells, namely the signal transduction mechanism.

The understanding of the molecular mechanism by which vascular endothelial cells respond to a shear stress leads us to learn the mechanism of development of various vascular diseases caused by arteriosclerosis, and the target for treatment. In order to elucidate the signal transduction mechanism, it is necessary to obtain a group of genes which exhibit a shear stress stimulus-dependent alteration of expression in vascular endothelial cells.

Moreover, the understanding of the molecular mechanism by which the apoptosis of vascular endothelial cells is suppressed in response to a shear stress stimulus leads us to elucidate the mechanism of the local formation of an early lesion of arteriosclerosis and thereby discover remedies for various vascular diseases caused by arteriosclerosis. In order to elucidate the molecular mechanism, it is necessary to obtain genes which exhibit a shear stress stimulus-dependent increase of expression in vascular endothelial cells and have an apoptosis-suppressing activity.

Summary of the Invention

The present inventors made intensive investigations with a view to solving the above-described problems and have now obtained the following results. Specifically, mRNA derived from cultured vascular endothelial cells having a shear stress applied thereto was used as a template to prepare a cDNA library, and mRNA extracted from endothelial cells having no shear stress applied thereto was subtracted therefrom. Thus, a subtraction library was constructed in which genes exhibiting an increase of expression under shear stress-applied conditions was concentrated. However, since abundance of genes having a low amount of

expression are equalized and empty vectors having no inserted fragment are increased in this subtraction library, a reverse subtraction method was newly developed to construct a second-generation subtraction library in which genes exhibiting an alteration of expression in response to a shear stress were concentrated from the subtraction library. Clones present in this second-generation subtraction library were randomly subjected to Northern hybridization, so that a large number of clones exhibiting an increase of expression by the application of a shear stress were obtained. Among these clones, not only the genes already known to exhibit an alteration of expression in response to a shear stress, but also genes presumed to act on the regulation of arteriosclerosis, genes which have not yet been known to be associated with arteriosclerosis, and novel genes were found. Furthermore, peptides encoded by these genes were found. Thus, the present invention has been completed.

Specifically, the present invention provides the following (1) to (76).

(1) A DNA having a nucleotide sequence selected from the nucleotide sequences represented by SEQ ID NO:143, 145, 147, 149, 151, 153, 155, 157, 168, 170 and 172.

(2) A shear stress-responsive DNA capable of hybridizing with a DNA having the nucleotide sequence represented by SEQ ID NO:143, 145, 149, 151, 153, 155, 157, 168, 170 or 172 under stringent conditions.

(3) A shear stress-responsive DNA capable of hybridizing with a DNA having the nucleotide sequence represented by SEQ ID NO:147 under stringent conditions, and having not less than 90% homology with the DNA.

(4) A DNA having the same sequence as 5 to 60 consecutive bases in a nucleotide sequence selected from the nucleotide sequences represented by SEQ ID NO:143, 145, 149, 153, 155, 157, 168, 170 and 172, or a DNA having a sequence complementary to the DNA.

(5) A method for detecting an mRNA for a shear stress-responsive gene using a DNA according to any of (1) to (4).

(6) A diagnostic agent for vascular diseases caused by arteriosclerosis which contains a DNA according to any of (1) to (4).

(7) A method for detecting a gene causative of arteriosclerosis using a DNA

according to any of (1) to (4).

(8) A method for screening an agent for regulating the transcription or translation of a shear stress-responsive gene using a DNA according to any of (1) to (4).

(9) A method for screening a therapeutic agent for vascular diseases caused by arteriosclerosis using a DNA according to any of (1) to (4).

(10) A therapeutic agent for vascular diseases caused by arteriosclerosis which contains a DNA according to any of (1) to (4).

(11) A recombinant virus vector containing a DNA according to any of (1) to (4).

(12) A recombinant virus vector containing an RNA comprising a sequence homologous with the sense strand of a DNA according to any of (1) to (4).

(13) A DNA having a nucleotide sequence selected from the nucleotide sequences represented by SEQ ID NO:111, 113, 115, 116, 117, 119, 121, 123, 125, 127, 129, 130, 131, 132, 133, 134, 135, 137, 139 and 141.

(14) A shear stress-responsive DNA capable of hybridizing with the DNA according to (13) under stringent conditions.

(15) A DNA having the same sequence as 5 to 60 consecutive bases in a nucleotide sequence selected from the nucleotide sequences represented by SEQ ID NO:111, 113, 115, 116, 117, 119, 121, 123, 125, 127, 129, 130, 131, 132, 133, 134, 135, 137, 139 and 141, or a DNA having a sequence complementary to the DNA.

(16) A diagnostic agent for vascular diseases caused by arteriosclerosis which contains a DNA according to any of (13) to (15).

(17) A method for detecting a gene causative of arteriosclerosis using a DNA according to any of (13) to (15).

(18) A method for screening an agent for regulating the transcription or translation of a shear stress-responsive gene using a DNA according to any of (13) to (15).

(19) A method for screening a therapeutic agent for vascular diseases caused by arteriosclerosis using a DNA according to any of (13) to (15).

(20) A method for detecting an mRNA for a shear stress-responsive gene using a

DNA having a nucleotide sequence selected from the nucleotide sequences represented by SEQ ID NO:1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107 and 109.

(21) A method for identifying the apoptosis sensitivity of cells by detecting the endogenous transcription level of a DNA having the nucleotide sequence represented by SEQ ID NO:7 using a DNA having the nucleotide sequence represented by SEQ ID NO:7 or a DNA having the same sequence as 5 to 60 consecutive bases in the nucleotide sequence represented by SEQ ID NO:7.

(22) A method for suppressing or promoting the apoptosis of cells by regulating the endogenous transcription or translation of a DNA having the nucleotide sequence represented by SEQ ID NO:7 using a DNA having the nucleotide sequence represented by SEQ ID NO:7 or a DNA having the same sequence as 5 to 60 consecutive bases in the nucleotide sequence represented by SEQ ID NO:7, or an antisense DNA having a nucleotide sequence complementary to the nucleotide sequence of each of these DNAs.

(23) A diagnostic agent for vascular diseases caused by arteriosclerosis which contains a DNA having a nucleotide sequence selected from the nucleotide sequences represented by SEQ ID NO:1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107 and 109.

(24) An agent for identifying the apoptosis sensitivity of cells which contains a DNA having the nucleotide sequence represented by SEQ ID NO:7, or a DNA having the same sequence as 5 to 60 consecutive bases in the nucleotide sequence represented by SEQ ID NO:7.

(25) A method for screening an agent for regulating the transcription or translation of a shear stress-responsive gene using a DNA having a nucleotide sequence selected from the nucleotide sequences represented by SEQ ID NO:1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75,

77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107 and 109.

(26) A method for screening a therapeutic agent for vascular diseases caused by arteriosclerosis using a DNA having a nucleotide sequence selected from the nucleotide sequences represented by SEQ ID NO:1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107 and 109.

(27) A method for screening an agent for suppressing or promoting the apoptosis of cells by regulating the endogenous transcription or translation of a DNA having the nucleotide sequence represented by SEQ ID NO:7 using a DNA having the nucleotide sequence represented by SEQ ID NO:7 or a DNA having the same sequence as 5 to 60 consecutive bases in the nucleotide sequence represented by SEQ ID NO:7.

(28) A therapeutic agent for vascular diseases caused by arteriosclerosis which contains a DNA having a nucleotide sequence selected from the nucleotide sequences represented by SEQ ID NO:1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107 and 109.

(29) An agent for suppressing or promoting the apoptosis of cells which contains a DNA having the nucleotide sequence represented by SEQ ID NO:7 or a DNA having the same sequence as 5 to 60 consecutive bases in the nucleotide sequence represented by SEQ ID NO:7, or an antisense DNA having a nucleotide sequence complementary to the nucleotide sequence of each of these DNAs.

(30) A recombinant virus vector containing a DNA having a nucleotide sequence selected from the nucleotide sequences represented by SEQ ID NO:1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107 and 109.

(31) A recombinant virus vector containing an RNA comprising a sequence homologous with the sense strand of a DNA having a nucleotide sequence selected from the nucleotide sequences represented by SEQ ID NO:1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25,

27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107 and 109.

(32) A therapeutic agent for vascular diseases caused by arteriosclerosis which contains a recombinant virus vector according to (30) or (31).

(33) A method for suppressing the apoptosis of cells using a recombinant virus vector containing a DNA having the nucleotide sequence represented by SEQ ID NO:7, or a recombinant virus vector containing an RNA comprising a sequence homologous with the sense strand of a DNA having the nucleotide sequence represented by SEQ ID NO:7.

(34) A method for screening an agent for suppressing or promoting the apoptosis of cells using a recombinant virus vector containing a DNA having the nucleotide sequence represented by SEQ ID NO:7, or a recombinant virus vector containing an RNA comprising a sequence homologous with the sense strand of a DNA having the nucleotide sequence represented by SEQ ID NO:7.

(35) A protein having an amino acid sequence selected from the amino acid sequences represented by SEQ ID NO:144, 146, 148, 150, 152, 154, 156, 158, 169, 171 and 173.

(36) A protein comprising an amino acid sequence in which one or more amino acids are deleted, replaced or added as compared with the amino acid sequence possessed by the protein according to (35), and having an activity participating in the formation of an arteriosclerotic lesion.

(37) A DNA encoding a protein according to (35) or (36).

(38) A recombinant DNA obtained by inserting a DNA according to any of (1)-(4) and (37) into a vector.

(39) A transformant obtained by introducing the recombinant DNA according to (38) into a host cell.

(40) A process for the preparation of a protein which comprises culturing the transformant according to (39) in a culture medium, causing a protein according to (35) or (36) to be produced and accumulated in the culture medium, and harvesting the protein from

the resulting culture.

(41) A method for screening a therapeutic agent for vascular diseases caused by arteriosclerosis which comprises culturing the transformant according to (39) in a culture medium and using the resulting culture for the screening.

(42) A method for screening a therapeutic agent for vascular diseases caused by arteriosclerosis using a protein according to (35) or (36).

(43) A recombinant virus vector capable of producing a protein according to (35) or (36).

(44) A therapeutic agent for vascular diseases caused by arteriosclerosis which contains the recombinant virus vector of (43).

(45) An antibody capable of recognizing a protein according to (35) or (36).

(46) A method for detecting a protein according to (35) or (36) immunologically using the antibody according to (45).

(47) A method for screening a therapeutic agent for vascular diseases caused by arteriosclerosis using the antibody according to (45).

(48) A method for screening an agent for regulating the transcription or translation of a shear stress-responsive gene using the antibody according to (45).

(49) A diagnostic agent for vascular diseases caused by arteriosclerosis which contains the antibody according to (45).

(50) A therapeutic agent for vascular diseases caused by arteriosclerosis which contains the antibody according to (45).

(51) A drug delivery method which comprises combining the antibody of (45) with a radioactive isotope, a protein or a low-molecular-weight agent, and delivering the resulting conjugated antibody to an arteriosclerotic lesion.

(52) An antibody capable of recognizing a protein having an amino acid sequence represented by SEQ ID NO:112, 114, 118, 120, 122, 124, 126, 128, 136, 138, 140 and 142.

(53) A method for screening a therapeutic agent for vascular diseases caused by arteriosclerosis using the antibody according to (52).

(54) A method for screening an agent for suppressing the transcription or translation of a shear stress-responsive gene using the antibody according to (52).

(55) A diagnostic agent for vascular diseases caused by arteriosclerosis which contains the antibody according to (52).

(56) A therapeutic agent for vascular diseases caused by arteriosclerosis which contains the antibody according to (52).

(57) A drug delivery method which comprises combining the antibody of (52) with a radioactive isotope, a protein or a low-molecular-weight agent, and directing the resulting conjugated antibody to an arteriosclerotic lesion.

(58) A method for screening an agent capable of binding specifically to a protein having the amino acid sequence represented by SEQ ID NO:8 and effective for suppressing or promoting the apoptosis of cells, using a protein having the amino acid sequence represented by SEQ ID NO:8.

(59) A method for screening an agent for suppressing or promoting the apoptosis of cells which comprises inserting a DNA having the nucleotide sequence represented by SEQ ID NO:7 or a DNA encoding a protein having the amino acid sequence represented by SEQ ID NO:8, into a vector; introducing the resulting recombinant DNA into a host cell; culturing the resulting transformant in a culture medium; and using the resulting culture for the screening.

(60) A recombinant virus vector capable of producing a protein having an amino acid sequence selected from the amino acid sequences represented by SEQ ID NO:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108 and 110.

(61) A therapeutic agent for vascular diseases caused by arteriosclerosis which contains the recombinant virus vector of (60).

(62) A method for suppressing the apoptosis of cells using a recombinant virus vector capable of producing a protein having the amino acid sequence represented by SEQ ID NO:8.

(63) An agent for suppressing the apoptosis of cells which contains a recombinant virus vector capable of producing a protein having the amino acid sequence represented by SEQ ID NO:8.

(64) A method for screening a therapeutic agent for vascular diseases caused by arteriosclerosis using an antibody capable of recognizing a protein having the amino acid sequence represented by SEQ ID NO:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108 or 110.

(65) A method for screening an agent for suppressing or promoting the transcription or translation of a shear stress-responsive gene using an antibody capable of recognizing a protein having the amino acid sequence represented by SEQ ID NO:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108 or 110.

(66) A method for regulating the apoptosis of cells using an antibody capable of recognizing a protein having the amino acid sequence represented by SEQ ID NO:8.

(67) A method for screening an agent for regulating the apoptosis of cells using an antibody capable of recognizing a protein having the amino acid sequence represented by SEQ ID NO:8.

(68) A method for identifying the apoptosis sensitivity of cells by detecting the expression level of a protein having the amino acid sequence represented by SEQ ID NO:8 using an antibody capable of recognizing a protein having the amino acid sequence represented by SEQ ID NO:8.

(69) A method according to any of (21), (22), (27), (33), (34), (58), (59), (62), (66), (67) and (68) wherein the cells are vascular endothelial cells.

(70) A diagnostic agent for vascular diseases caused by arteriosclerosis which contains an antibody capable of recognizing a protein having the amino acid sequence represented by SEQ ID NO:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88,

90, 92, 94, 96, 98, 100, 102, 104, 106, 108 or 110.

(71) An agent for identifying the apoptosis sensitivity of cells which contains an antibody capable of recognizing a protein having the amino acid sequence represented by SEQ ID NO:8.

(72) A therapeutic agent for vascular diseases caused by arteriosclerosis which contains an antibody capable of recognizing a protein having the amino acid sequence represented by SEQ ID NO:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108 or 110.

(73) An agent for regulating the apoptosis of cells which comprises an antibody capable of recognizing a protein having the amino acid sequence represented by SEQ ID NO:8.

(74) An agent for suppressing or promoting the apoptosis of cells which is obtained by a method according to any of (27), (34), (58), (59) and (67).

(75) An agent according to any of (24), (29), (63), (71), (73) and (74) wherein the cells are vascular endothelial cells.

(76) A drug delivery method which comprises combining an antibody capable of recognizing a protein having the amino acid sequence represented by SEQ ID NO:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108 or 110, with a radioactive isotope, a protein or a low-molecular-weight agent, and directing the resulting conjugated antibody to an arteriosclerotic lesion.

The term "regulate" as used herein means the action of suppressing or promoting. Moreover, the term "agent" refers to any substances having an arbitrary molecular weight such as proteins and nucleic acids.

The DNA of the present invention is a shear stress-responsive DNA. Examples thereof include a DNA having a nucleotide sequence selected from the nucleotide sequences represented by SEQ ID NO:143, 145, 147, 149, 151, 153, 155, 157, 168, 170 and 172; and a

DNA capable of hybridizing with the foregoing DNAs under stringent conditions and showing an alteration in the expression level in response to the application of a shear stress.

The above-described DNA capable of hybridizing with a nucleotide sequence selected from the nucleotide sequences represented by SEQ ID NO:143, 145, 147, 149, 151, 153, 155, 157, 168, 170 and 172 under stringent conditions is a DNA obtained by carrying out colony hybridization, plaque hybridization or Southern blot hybridization while using a DNA having a nucleotide sequence selected from the nucleotide sequences represented by SEQ ID NO:143, 145, 147, 149, 151, 153, 155, 157, 168, 170 and 172 as a probe. Specifically, it includes DNA which can be identified by using a filter having colony- or plaque-derived DNAs immobilized thereon to carry out hybridization at 65°C in the presence of 0.7-1.0 M NaCl, and then washing the filter with an SSC solution having a 0.1- to 2-fold concentration (an SSC solution having a one-fold concentration is composed of 150 mM sodium chloride and 15 mM sodium citrate) under 65°C conditions.

Hybridization may be carried out according to the methods described in Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press (1989) (hereinafter referred to briefly as "Molecular Cloning, Second Edition"); Current Protocols in Molecular Biology, John Wiley & Sons (1987-1997) (hereinafter referred to briefly as "Current Protocols in Molecular Biology"); DNA Cloning 1: Core Techniques, A Practical Approach, Second Edition, Oxford University (1995); and the like. Specific examples of the hybridizable DNAs include DNAs having not less than 60% homology, preferably not less than 80% homology, more preferably not less than 90% homology, and most preferably not less than 95% homology with a nucleotide sequence selected from the nucleotide sequences represented by SEQ ID NO:143, 145, 147, 149, 151, 153, 155, 157, 168, 170 and 172.

Furthermore, the DNA of the present invention also includes oligonucleotides and antisense oligonucleotide having a sequence of a part of the DNA of the present invention. The oligonucleotide includes, for example, an oligonucleotide having the same sequence as the nucleotide sequence of 5 to 60 residues, preferably 10 to 40 residues, in a nucleotide sequence selected from the nucleotide sequences represented by SEQ ID NO:143, 145, 147,

149, 151, 153, 155, 157, 168, 170 and 172. The antisense oligonucleotide includes, for example, an antisense oligonucleotide of the foregoing oligonucleotide.

The protein of the present invention includes a protein having an activity associated with arteriosclerosis. Specific examples thereof include a protein having an amino acid sequence selected from the amino acid sequences represented by SEQ ID NO:144, 146, 148, 150, 152, 154, 156, 158, 169, 171 and 173, and a protein comprising amino acid sequences in which one or more amino acids are deleted, replaced or added as compared with the amino acid sequence possessed by the foregoing protein, and having an activity involved in the formation of an arteriosclerotic lesion.

The protein comprising amino acid sequence in which one or more amino acids are deleted, replaced or added as compared with the amino acid sequence of protein having an amino acid sequence selected from the amino acid sequences represented by SEQ ID NO:144, 146, 148, 150, 152, 154, 156, 158, 169, 171 and 173, and having an activity involved in the formation of an arteriosclerotic lesion may be prepared according to the methods described in Molecular Cloning, Second Edition; Current Protocols in Molecular Biology; Nucleic Acids Research, 10, 6487(1982); Proc. Natl. Acad. Sci. USA, 79, 6409(1982); Gene, 34, 315(1985); Nucleic Acids Research, 13, 4431(1985); Proc. Natl. Acad. Sci. USA, 82, 488(1985); and the like.

Moreover, among the acquired large number of genes exhibiting an increase of expression by the application of a shear stress in vascular endothelial cells, the present inventors have found A4RS-041 having homology with LFG (lifeguard), a brain-specific gene which has been reported to suppress Fas-mediated apoptosis [Proc. Natl. Acad. Sci. USA, 22, 12673-12678(1999)]. First of all, according to an analysis of the nucleotide sequence of A4RS-041, the present inventors have found that A4RS-041 is a gene entirely different from LFG because A4RS-041 has about 50% identity to LFG, but about one-third thereof on the amino-terminal side has little homology. Moreover, the present inventors have also found that the expression profiles of A4RS-041 and LFG in tissues are substantially different because A4RS-041 is widely expressed in a variety of tissues including vascular endothelial

cells, whereas LFG is highly expressed in the brain but not in vascular endothelial cells. Furthermore, by constructing a transformed cell which permits A4RS-041 to be stably and highly expressed, the present inventors have also found that A4RS-041 suppresses Fas-mediated apoptosis, thus ascertaining that A4RS-041 is a key molecule for the suppression of the apoptosis of vascular endothelial cells by a shear stress. Thus, the present invention has been completed.

Brief Description of the Drawings

FIG. 1 illustrates the results of Northern analysis of genes exhibiting an increase of expression in response to a shear stress stimulus. Lanes 1-41 show shear stress-dependent increases of expression for A4RS-016, -026, -040, -041, -063, -096, -116, -126, -131, -148, -154, -174, -175, -194, -197, -260, -271, -307, -355, -389, -391, -423, -431, -453, -492, -507, -514, -523, -544, -547, -557, -577, -588, -602, -608, -612, -625, -666, -668, -674 and -682, respectively. In each blot, 4 μ g of total RNA derived from HUVEC having no shear stress applied thereto (with a stimulation time of 0) was electrophoresed in the left-hand lane, and 4 μ g of total RNA derived from HUVEC having a shear stress applied thereto (a mixture of equal amounts of total RNA samples derived from HUVEC stimulated for 0.5, 1, 1.5, 2, 3, 4, 6, 10 and 20 hours) was electrophoresed in the right-hand lane.

FIG. 2 illustrates the results of Northern analysis of genes exhibiting an increase of expression in response to a shear stress stimulus. Lanes 42-83 show shear stress-dependent increases of expression for A4RS-751, -781, -784, -817, -818, -914, -929, -935, -938, -939, -945, -947, -948, -949, -011, -115, -143, -171, -193, -280, -402, -533, -604, -615, -619, -626, -676, -679, -737, -780, -826, -916, -933, -943, -002, -049, -230, -239, -242, -491, -578 and -829, respectively. In each blot, 4 μ g of total RNA derived from HUVEC having no shear stress applied thereto (with a stimulation time of 0) was electrophoresed in the left-hand lane, and 4 μ g of total RNA derived from HUVEC having a shear stress applied thereto (a mixture of equal amounts of total RNA samples derived from HUVEC stimulated for 0.5, 1, 1.5, 2, 3, 4, 6, 10 and 20 hours) was electrophoresed in the right-hand lane.

FIG. 3 illustrates the results of Northern blotting analysis of genes expressed in

response to a shear stress stimulus, showing their changes of expression with time. Lanes 1-17 show shear stress-dependent increases of expression for A4RS-016, -041, -063, -096, -116, -260, -271, -307, -389, -391, -602, -784, -115, -143, -193, -280 and -402, respectively. In each blot, 4 μ g of total RNA samples derived from HUVEC having shear stress application times of 0, 0.5, 1, 1.5, 2, 3, 4, 6, 10 and 20 hours respectively were electrophoresed as viewed from left to right.

FIG. 4 illustrates the results of Northern blotting analysis of genes expressed in response to a shear stress stimulus, showing their changes of expression with time. Lanes 18-28 show shear stress-dependent increases of expression for A4RS-604, -626, -916, -002, -049, -230, -239, -242, -491, -578 and -829, respectively. In each blot, 4 μ g of total RNA samples derived from HUVEC having shear stress application times of 0, 0.5, 1, 1.5, 2, 3, 4, 6, 10 and 20 hours respectively were electrophoresed as viewed from left to right.

FIG. 5 illustrates the construction of the plasmid pAMo-002 for the expression in animal cells.

FIG. 6A and FIG. 6B are diagrams showing the apoptosis suppressing activity of A4RS-041. FIG. 6A shows changes with time when the anti-Fas monoclonal antibody concentration was fixed at 100 ng/ml, and FIG. 6B shows dependence on the anti-Fas monoclonal antibody concentration when the stimulation time was fixed at 36 hours. ● represents HeLa cells into which A4RS-041 was introduced, and ■ represents HeLa cells into which GFP was introduced.

FIG. 7A and FIG. 7B are diagrams showing the distribution of expression of A4RS-041. FIG. 7A is a diagram showing the results obtained by analyzing the expression of A4RS-041 in human normal tissues by Northern blotting. FIG. 7B is a diagram showing the results obtained by analyzing the expression of A4RS-041 and LFG in human vascular endothelial cells and human brain by RT-PCR.

FIG. 8 is a diagram showing the amino acid sequence homology of A4RS-041 and LFG.

Detailed Description of the Invention

The present invention will be more specifically described hereinbelow. No particular limitation is placed on the type of cells used to prepare the DNA of the present invention, so long as they exhibit responsiveness to the application of a shear stress. However, adhesion type cells are preferred. Examples thereof include vascular endothelial cells, and human vascular endothelial cells are especially preferred. More preferred are human umbilical vein endothelial cells (HUVECs). These vascular endothelial cells can be easily separated from a human umbilical cord according to the method described in Cell (in Japanese), 20, 329(1988) or Human Cell, 1, 188(1988). It is also possible to obtain and use secondary cultured cells having been separated. The passage number of vascular endothelial cells is not critical, provided that they retain properties as vascular endothelial cells. However, vascular endothelial cells having a passage number of 20 or less are preferred.

The culture medium used for cell culture may have a conventionally known composition. In the case, for example, of vascular endothelial cells, it is preferable to use a cell culture medium to which 0 to 20% of the blood serum of an animal such as cattle is added. More preferred is E-GM medium (containing 2% fetal calf serum; manufactured by Kurabo Industries, Ltd.) or M199 medium having 20% fetal calf serum added thereto. In order to promote the growth of cells, a cell growth factor such as ECGS (endothelial cell growth supplement), EGF (epidermal growth factor) or basic FGF (fibroblast growth factor) may be added to the culture medium. A high shear stress can be applied to the cultured cells by adding dextran or the like to the culture medium and thereby increasing the viscosity of the culture medium.

As the culture apparatus permitting the application of a shear stress, there may be used an apparatus of the micro-carrier type [Am. J. Physiol., 259, H804(1990)], the rotary disc type [Biorheology, 25, 461(1988)], the parallel plate type [Biotechnol. Bioeng., 27, 1021(1985)] or the like.

In the application of a shear stress, no particular limitation is placed on the method for the culture of vascular endothelial cells. One exemplary method is as follows. Vascular endothelial cells are allowed to adhere to micro-carriers and suspended in a culture medium

within a spinner flask. Although the incubation temperature may be any desired temperature that permit the culture of the cells, it is preferable to use a temperature of 37°C. The incubation is preferably carried out in an incubator filled with 5% carbon dioxide gas. No particular limitation is placed on the number of cells harvested, so long as RNA can be extracted therefrom. A typical example thereof is a number of that order which can be obtained by ordinary culture techniques, and a number of not less than 1×10^6 cells is preferred. Although the incubation time is not specified, it is preferable to use an incubation time at which the expression of a gene is distinctly changed as compared with a culture without the application of a shear stress. Especially preferred is an incubation time which provides good viability of the cells. Specifically, an incubation time in the range of 4 to 24 hours is useful.

As the method for preparing total RNA from vascular endothelial cells having a shear stress applied thereto, the guanidine thiocyanate-cesium trifluoroacetate method [Methods in Enzymol., 154, 3(1987)] or the like may be employed.

As the method for preparing poly(A)⁺ RNA from total RNA, the oligo(dT)-immobilized cellulose column method (Molecular Cloning, Second Edition) or the like may be employed.

Furthermore, mRNA may be prepared by using a kit such as Fast Track mRNA Isolation Kit (manufactured by Invitrogen) or Quick Prep mRNA Purification Kit (manufactured by Amersham Pharmacia Biotech).

Now, the method for the construction of a cDNA library is described below. Usable methods for the construction of a cDNA library include the methods described in Molecular Cloning, Second Edition, Current Protocols in Molecular Biology, DNA Cloning 1: Core Techniques, A Practical Approach, Second Edition, Oxford University Press (1995), and the like; and methods using a commercially available kit such as SuperScript Plasmid System for cDNA Synthesis and Plasmid Cloning (manufactured by Life Technologies) or ZAP-cDNA Synthesis Kit (manufactured by Stratagene).

As a cloning vector for the construction of a cDNA library, there may be used any of phage vectors, plasmid vectors and the like, provided that they can replicate autonomously in

Escherichia coli K12 strain. Specific examples thereof include ZAP Express [manufactured by Stratagene; Strategies, 5, 58(1992)], pBluescript II SK(+) [Nucleic Acids Res., 17, 9494(1989)], λ zap II (manufactured by Stratagene), λ gt10, λ gt11 [DNA Cloning, A Practical Approach, 1, 49(1985)], λ BlueMid (manufactured by Clontech), λ ExCell (manufactured by Amersham Pharmacia Biotech), pT7T318U (manufactured by Amersham Pharmacia Biotech), pcD2 [Mol. Cell. Biol., 3, 280(1983)] and pUC18 [Gene, 33, 103(1985)].

As a Escherichia coli for introducing vectors having cDNAs integrated therein, there may be used any microorganism that belongs to Escherichia coli. Specifically, Escherichia coli XL1-Blue MRF' [manufactured by Stratagene; Strategies, 5, 81(1992)], Escherichia coli C600 [Genetics, 39, 440(1954)], Escherichia coli Y1088 [Science, 222, 778(1983)], Escherichia coli Y1090 [Science, 222, 778(1983)], Escherichia coli NM522 [J. Mol. Biol., 166, 1(1983)], Escherichia coli K802 [J. Mol. Biol., 16, 118(1966)], Escherichia coli JM105 [Gene, 38, 275(1985)] and the like may be used.

Since this cDNA library has the characteristics of vascular endothelial cells having a shear stress applied thereto, it is useful, for example, in cloning a gene associated with a lesion occurring in vital vascular regions undergoing a change of shear stress (specifically, the formation of arteriosclerotic lesions, or the like) and in developing pharmaceuticals by controlling the expression of the gene. Moreover, this cDNA library is different in the types and quantities of genes contained therein, from a cDNA library constructed by using mRNA derived from another type of cells (specifically, standing-cultured vascular endothelial cells having no shear stress applied thereto) as a template. Accordingly, it is possible to isolate the above-described gene associated with the formation of arteriosclerotic lesions or a protein encoded by the gene while using the difference as an index.

As the method for concentrating genes exhibiting an increase of expression by the application of a shear stress from the cDNA library so constructed, there may be employed a method such as the subtraction method [Proc. Natl. Acad. Sci. USA, 88, 2825(1991)] or differential hybridization [J. Biol. Chem., 265, 2973(1990)].

As the method for selecting clones having expression specificity (i.e., exhibiting an

increase of expression by the application of a shear stress) from the subtraction library in which such genes are concentrated in the above-described manner, there may be employed Northern hybridization [Molecular Cloning, Second Edition], RT(reverse-transcribed)-PCR [Current Protocols in Molecular Biology] or the like.

With respect to the shear stress-responsive clone selected in the above-described manner, the nucleotide sequence of the DNA can be determined by analyzing it according to a commonly employed nucleotide sequence analysis method such as the dideoxy method of Sanger et al. [Proc. Natl. Acad. Sci. USA, 74, 5463(1977)], or by means of a nucleotide sequence analyzer such as 373A DNA Sequencer (manufactured by Perkin Elmer).

The novelty of the nucleotide sequence determined in the above-described manner can be confirmed by using a homology search program (e.g., blast) to search the nucleotide sequence in nucleotide sequence databases such as GenBank, EMBL and DDBJ, and thereby ascertaining that the databases do not include any nucleotide sequence having a distinct identity to the aforesaid nucleotide sequence and hence considered to be identical thereto.

When the DNA obtained in the above-described manner is a partial DNA of the cDNA corresponding to a shear stress-related mRNA, a clone containing the full-length cDNA may be selected again from the cDNA library by using the DNA obtained in the above-described manner as a probe.

The selection of a cDNA clone from the cDNA library may be carried out by colony hybridization or plaque hybridization using a probe labeled with an isotope or digoxigenin [Sambrook et al., Molecular Cloning, Second Edition (1989)].

Examples of the full-length cDNA of the shear stress-responsive gene having a novel nucleotide sequence, which is obtained in the above-described manner, include DNAs having the nucleotide sequences represented by SEQ ID NO:143, 145, 147, 149, 151, 153, 155, 157, 168, 170 and 172.

Once the full-length cDNA of a shear stress-related gene is obtained and its nucleotide sequence is determined as described above, the desired DNA can be obtained by preparing primers based on the nucleotide sequence and carrying out PCR [PCR Protocols, Academic

Press (1990)] while using cDNA synthesized from mRNA or a cDNA library as a template. Moreover, the desired DNA may also be prepared by using a DNA synthesizer to synthesize it chemically on the basis of the determined nucleotide sequence of the DNA. Usable DNA synthesizers include Model 392 DNA Synthesizer (manufactured by Perkin Elmer) using the phosphoamidite method, and the like.

On the basis of the nucleotide sequence information of the aforesaid DNA and DNA fragment, an oligonucleotide and an antisense oligonucleotide each having a partial sequence of the DNA of the present invention may be prepared according to a conventional method or by means of a DNA synthesizer.

Examples of the oligonucleotide or antisense oligonucleotide include a sense primer corresponding to a nucleotide sequence on the 5'-terminal side and an antisense primer corresponding to a nucleotide sequence on the 3'-terminal side, both in a partial nucleotide sequence of mRNA to be detected. However, the base corresponding to uracil in mRNA is thymidine in oligonucleotide primers. Preferably, the sense primer and antisense primer are oligonucleotides whose melting temperatures (T_m) and numbers of bases are not extremely different from each other and which consist of 10 to 40 bases.

Moreover, in the present invention, there may be used derivatives of the nucleotides. Examples thereof include methyl derivatives and phosphothioate derivatives of the nucleotides.

Now, the method for the preparation of a protein having an activity involved in the formation of an arteriosclerotic lesion is described below.

The cDNA of the shear stress-responsive gene, which was obtained in the above-described manner, encodes a protein having an activity involved in the formation of an arteriosclerotic lesion.

The activity involved in the formation of an arteriosclerotic lesion means an activity regulating the development of arteriosclerosis, and preferably an activity preventing the development of arteriosclerosis. Examples thereof include, but are not limited to, the following activities.

They include regulation of the incorporation of low-density lipoprotein (LDL) into the vascular endothelium; regulation of the incorporation of oxidized LDL into the vascular endothelium; regulation of the expression of LDL receptors in vascular endothelial cells; regulation of the production of oxidized LDL in vascular endothelial cells; regulation of the expression of scavenger receptors in the vascular endothelium; regulation of the infiltration of lymphocytes into blood vessels; regulation of the expression of a cell surface adhesion molecule promoting the infiltration of lymphocytes into blood vessels in vascular endothelial cells; regulation of the proliferation of vascular smooth muscle produced in vascular endothelial cells; regulation of the apoptosis of vascular endothelial cells; and the like.

The DNAs and proteins of the present invention have been found on the basis of their shear stress-dependent increase of expression in vascular endothelial cells. As described in the Background of the Invention, it is generally known that arteriosclerosis occurs frequently in places where a low shear stress is produced and the separation or stagnation of a flow or turbulence (e.g., eddies) tends to occur. Accordingly, the DNAs and proteins of the present invention are especially useful for the treatment or prevention of arteriosclerosis or various vascular diseases caused thereby, including non-limitative examples such as cardiac insufficiency, restenosis after PTCA, and hypertension.

If necessary, a DNA fragment of appropriate length containing a portion encoding the protein is prepared on the basis of the full-length cDNA.

An expression plasmid for the protein is created by inserting the DNA fragment or the full-length cDNA into an expression vector on the downstream side of a promoter.

The expression plasmid is introduced into a host cell suited to the expression vector.

As the host cell, there may be used any host cell that enables the expression of the desired DNA. For example, there may be used bacteria belonging to the genera Escherichia, Serratia, Corynebacterium, Brevibacterium, Pseudomonas, Bacillus and Microbacterium; yeasts belonging to the genera Kluyveromyces, Saccharomyces, Shizosaccharomyces, Trichosporon and Schawnniomyces; animal cells; and insect cells.

As the expression vector, there is used a vector which can be autonomously replicated

or incorporated into a chromosome in the aforesaid host cell and which contains a promoter at a position capable of transcribing the shear stress-responsive DNA.

When a bacterium or the like is used as the host cell, the shear stress-responsive DNA expression vector is preferably a recombinant vector which can be autonomously replicated in the bacterium and which consists of a promoter, a ribosome-binding sequence, the shear stress-responsive DNA and a transcription termination sequence. A gene controlling the promoter may be contained therein.

Examples of such expression vectors include pBTrp2, pBTac1, pBTac2 (all commercially available from Boehringer Mannheim), pKK233-2 (manufactured by Amersham Pharmacia Biotech), pSE280 (manufactured by Invitrogen), pGEMEX-1 (manufactured by Promega), pQE-8 (manufactured by QIAGEN), pKYP10 (Japanese Published Unexamined Patent application No. 110600/83), pKYP200 [Agricultural Biological Chemistry, 48, 669 (1984)], pLSA1 [Agric. Biol. Chem., 53, 277(1989)], pGEL1 [Proc. Natl. Acad. Sci. USA, 82, 4306(1985)], pBluescript II SK(-) (manufactured by Stratagene), pGEX (manufactured by Amersham Pharmacia Biotech), pET-3 (manufactured by Novagen), pTerm2 (USP 4686191, USP 4939094, USP 5160735), pSupex, pUB110, pTP5, pC194, pEG400 [J. Bacteriol., 172, 2392(1990)].

The promoter may be any promoter that can express a gene in the host cell. Examples thereof include promoters derived from Escherichia coli and phages, such as trp promoter (Ptrp), lac promoter (Plac), P_L promoter, P_R promoter and T7 promoter; and SP01 promoter, SP02 promoter and penP promoter. Moreover, there may also be used artificially designed or modified promoters and the like, such as a promoter comprising two Ptrp's connected in series (Ptrpx2), tac promoter, letI promoter [Gene, 44, 29(1986)] and lacT7 promoter.

The ribosome-binding sequence may be any ribosome-binding sequence that can be expressed in the host cell. However, it is preferable to use a plasmid in which the distance between the Shine-Dargarno sequence and the initiation codon is adjusted to a suitable length (e.g., 6-18 bases).

In the nucleotide sequence of the protein-encoding part of the shear stress-responsive

DNA of the present invention, some residues may be replaced so as to give the codons most suitable for its expression in the host. Thus, the production rate of the desired protein can be improved.

A transcription termination sequence is not necessarily required for the expression of the shear stress-responsive DNA of the present invention. However, it is desirable to dispose a transcription termination sequence just downstream of the structural gene.

Examples of the host cell include microorganisms belonging to the genera Escherichia, Serratia, Corynebacterium, Brevibacterium, Pseudomonas, Bacillus and the like, such as Escherichia coli XL1-Blue, Escherichia coli XL2-Blue, Escherichia coli DH1, Escherichia coli MC1000, Escherichia coli KY3276, Escherichia coli W1485, Escherichia coli JM109, Escherichia coli HB101, Escherichia coli No.49, Escherichia coli W3110, Escherichia coli NY49, Bacillus subtilis, Bacillus amyloliquefaciens, Brevibacterium ammoniagenes, Brevibacterium immariophilum ATCC14068, Brevibacterium saccharolyticum ATCC14066, Corynebacterium glutamicum ATCC13032, Corynebacterium glutamicum ATCC14067, Corynebacterium glutamicum ATCC13869, Corynebacterium acetoacidophilum ATCC13870, Microbacterium ammoniaphilum ATCC15354, and Pseudomonas sp. D-0110.

As the method for introducing the recombinant vector, there may be employed any method that can introduce DNA into the aforesaid host cell. Examples thereof include the calcium ion method [Proc. Natl. Acad. Sci. USA, 69, 2110(1972)], the protoplast method (Japanese Published Unexamined Patent Application No. 248397/88), and the methods described in Gene, 17, 107(1982) and Molecular & General Genetics, 168, 111(1979).

Where a yeast is used as the host cell, usable expression vectors include, for example, YEp13 (ATCC37115), YEp24 (ATCC37051), YCp50 (ATCC37419), pHS19 and pHS15.

The promoter may be any promoter that can express a gene in the yeast. Examples thereof include PHO5 promoter, PGK promoter, GAP promoter, ADH promoter, gal 1 promoter, gal 10 promoter, heat shock protein promoter, MFa1 promoter and CUP 1 promoter.

Examples of the host cell include Saccharomyces cerevisiae, Shizosaccharomyces pombe, Kluyveromyces lactis, Trichosporon pullulans and Schwanniomyces alluvius.

As the method for introducing the recombinant vector, there may be employed any method that can introduce DNA into yeasts. Examples thereof include the electroporation method [Methods. Enzymol., 194, 182(1990)], the spheroplast method [Proc. Natl. Acad. Sci. USA, 75, 1929(1978)], the lithium acetate method [J. Bacteriol., 153, 163(1983)], and the methods described in Proc. Natl. Acad. Sci. USA, 75, 1929(1978).

Where an animal cell is used as the host cell, usable expression vectors include, for example, pcDNAI, pcDM8 (manufactured by Funakoshi), pAGE107 [Japanese Published Unexamined Patent Application No. 22979/90; Cytotechnology, 3, 133(1990)], pAS3-3 (Japanese Published Unexamined Patent Application No. 227075/90), pCDM8 [Nature, 329, 840(1987)], pcDNAI/Amp (manufactured by Invitrogen), pREP4 (manufactured by Invitrogen), pAGE103 [J. Biochem., 101, 1307(1987)] and pAGE210.

The promoter may be any promoter that can be expressed in the animal cell. Examples thereof include the promoter of the IE (immediate early) gene of cytomegalovirus (human CMV), the early promoter of SV40, the promoter of retroviruses, metallothionein promoter, heat shock protein promoter, and SR α promoter. The enhancer of the IE gene of human CMV may be used in conjunction with the promoter.

Examples of the host cell include Namalwa cell derived from a human, COS cell derived from a monkey, CHO cell derived from a Chinese hamster, and HBT5637 (Japanese Published Unexamined Patent Application No. 299/88).

As the method for introducing the recombinant vector into the animal cell, there may be employed any method that can introduce DNA into animal cells. Examples thereof include the electroporation method [Cytotechnology, 3, 133(1990)], the calcium phosphate method (Japanese Published Unexamined Patent Application No. 227075/90), the lipofection method [Proc. Natl. Acad. Sci. USA, 84, 7413(1987)], and the methods described in Virology, 52, 456(1973). Transformants may be harvested and cultured according to the method described in Japanese Published Unexamined Patent Application No. 227075/90 or 257891/90.

Where an insect cell is used as the host, the protein may be expressed according to the method described, for example, in Baculovirus Expression Vectors, A Laboratory Manual,

Current Protocols in Molecular Biology Supplement 1-38 (1987-1997), or Bio/Technology, 6, 47(1988).

Specifically, a recombinant gene transfer vector and a baculovirus are co-introduced into an insect cell. After a recombinant virus is obtained in the culture supernatant of the insect cell, an insect cell is further infected with the recombinant virus to express the protein.

Examples of the gene transfer vector used in this method include pVL1392, pVL1393 and pBlueBacIII (all manufactured by Invitrogen).

As the baculovirus, there may be used, for example, autographa californica nuclear polyhedrosis virus that is a virus infecting insects belonging to Noctuidae.

As the insect cell, there may be used Sf9 and Sf21 that are ovarian cells of Spodoptera frugiperda [Baculovirus Expression Vectors, A Laboratory Manual, W.H. Freeman and Company, New York (1992)], High 5 that is an ovarian cell of Trichoplusia ni (manufactured by Invitrogen), and the like.

The methods which may be employed for co-introducing the aforesaid recombinant gene transfer vector and the aforesaid baculovirus into an insect cell in order to prepare a recombinant virus include, for example, the calcium phosphate method (Japanese Published Unexamined Patent Application No. 227075/90) and the lipofection method [Proc. Natl. Acad. Sci. USA, 84, 7413(1987)].

The expression of the gene may be effected not only by direct expression, but also by secretory production, fusion protein expression or the like, for example, according to the methods described in Molecular Cloning, Second Edition.

When the gene is expressed by means of a yeast, an animal cell or an insect cell, the protein having a sugar or sugar chain added thereto may be obtained.

A shear stress-responsive protein may be prepared by culturing a transformant containing a recombinant DNA having the shear stress-responsive DNA integrated therein into a culture, causing a shear stress-responsive protein to be produced and accumulated in the culture, and harvesting the protein from the resulting culture.

In order to culture the transformant of the present invention for the preparation of the

shear stress-responsive protein in a culture medium, there may be employed a common method for the culture of the host.

When the transformant of the present invention is a procaryote (e.g., Escherichia coli) or a eucaryote (e.g., yeast), the culture medium for the culture of such microorganisms may be a natural medium or a synthetic medium, provided that this medium contains a carbon source, a nitrogen source, minerals and other nutrients which can be assimilated by the microorganism and that this medium permits the transformant to be efficiently cultured.

The carbon source may be any carbon source that can be assimilated by the respective microorganisms. There may be used carbohydrates such as glucose, fructose, sucrose, molasses containing them, starch and starch hydrolyzate; organic acids such as acetic acid and propionic acid; and alcohols such as ethanol and propanol.

As the nitrogen source, there may be used ammonia; ammonium salts of various inorganic acids or organic acids, such as ammonium chloride, ammonium sulfate, ammonium acetate and ammonium phosphate; and other nitrogen-containing compounds, as well as peptone, meat extract, yeast extract, corn steep liquor, casein hydrolyzate, soybean meal and soybean meal hydrolyzate, various fermented bacterial cells and their digestion products, and the like.

As the minerals, there may be used potassium dihydrogen phosphate, dipotassium hydrogen phosphate, magnesium phosphate, magnesium sulfate, sodium chloride, ferrous sulfate, manganese sulfate, copper sulfate, calcium carbonate and the like.

The cultivation is carried out under aerobic conditions, for example, according to a shaking culture or deep aerated spinner culture technique. The incubation temperature should be in the range of 15 to 40°C and the incubation time usually ranges from 16 hours to 7 days. During cultivation, pH is maintained at 3.0 to 9.0. The adjustment of pH is made with an inorganic or organic acid, an alkaline solution, urea, calcium carbonate, ammonia or the like.

During cultivation, an antibiotic such as ampicillin or tetracycline may be added to the culture medium, if necessary.

When a microorganism transformed with an expression vector using an inducible

promoter is cultured, an inducer may be added to the culture medium, if necessary. For example, when a microorganism transformed with an expression vector using lac promoter is cultured, isopropyl- β -D-thiogalactopyranoside (IPTG) or the like may be added to the culture medium, and when a microorganism transformed with an expression vector using trp promoter is cultured, indoleacrylic acid (IAA) or the like may be added to the culture medium.

As the culture medium for culturing a transformant obtained by using an animal cell as the host cell, there may be used any of commonly used culture media such as RPMI1640 medium [The Journal of the American Medical Association, 199, 519(1967)], Eagle's MEM [Science, 122, 501(1952)], Dulbecco-modified MEM [Virology, 8, 396(1959)], 199 medium [Proceeding of the Society for the Biological Medicine, 73, 1(1950)], and culture media prepared by adding fetal calf serum or the like to the foregoing media.

The cultivation is usually carried out for 1 to 7 days under conditions including a pH of 6 to 8, a temperature of 30 to 40°C, and the presence of 5% CO₂.

During cultivation, an antibiotic such as kanamycin or penicillin may be added to the culture medium, if necessary.

As the culture medium for culturing a transformant obtained by using an insect cell as the host cell, there may be used any of commonly used culture media such as TNM-FH medium (manufactured by Pharmingen), Sf-900 II SFM medium (manufactured by Life Technologies), ExCel1400, ExCel1405 (both manufactured by JRH Biosciences), and Grace's Insect Medium [Nature, 195, 788(1962)].

The cultivation is usually carried out for 1 to 5 days under conditions including a pH of 6 to 7 and a temperature of 25 to 30°C.

During cultivation, an antibiotic such as gentamicin may be added to the culture medium, if necessary.

In order to isolate and purify a protein having an activity associated with arteriosclerosis in accordance with the present invention, from the culture of the transformant of the present invention, there may be employed common techniques for the isolation and purification of enzymes.

For example, where the protein of the present invention is expressed in a dissolved state within cells, the cells are collected by centrifugation after completion of the incubation, suspended in an aqueous buffer, and disrupted with a sonicator, French press, Manton Gaulin homogenizer, Dynamill or the like to obtain a cell-free extract. From the supernatant obtained by centrifuging the cell-free extract, a purified preparation may be obtained by employing common techniques for the isolation and purification of enzymes, either alone or in combination. These techniques include, for example, solvent extraction, salting-out with ammonium sulfate or the like, desalting, precipitation with an organic solvent, anion-exchange chromatography using a resin such as diethylaminoethyl (DEAE)-Sepharose or DIAION HPA-75 (manufactured by Mitsubishi Chemical Corp.), cation-exchange chromatography using a resin such as S-Sepharose FF (manufactured by Amersham Pharmacia Biotech), hydrophobic chromatography using a resin such as butyl Sepharose or phenyl Sepharose, gel filtration with a molecular sieve, affinity chromatography, chromatofocusing, and electrophoresis such as isoelectric focusing.

Where the protein is expressed in the form of an insoluble material within cells, the cells are collected, disrupted and centrifuged. Thus, the insoluble protein is recovered as a precipitate fraction.

The insoluble protein so recovered is solubilized with a protein denaturing agent.

The solubilized solution is diluted or dialyzed to reduce the concentration of the protein denaturing agent in the solubilized solution and thereby refold the protein to a normal stereostructure. Thereafter, a purified preparation of the protein may be obtained according to the same isolation and purification techniques as described above.

Where the protein of the present invention or a derivative thereof (e.g., a glycosylated product) is secreted out of cells, the protein or a derivative thereof (e.g., a glycosylated product) may be recovered from the culture supernatant. Specifically, the culture supernatant is recovered from the resulting culture according to a technique such as centrifugation. Then, a purified preparation may be obtained from the culture supernatant by employing the same isolation and purification techniques as described above.

Examples of the protein thus obtained include proteins having the amino acid sequences represented by SEQ ID NO:144, 146, 148, 150, 152, 154, 156, 158, 169, 171, 173 and the like.

The protein expressed in the above-described manner may also be prepared by chemical synthesis processes such as Fmoc method (fluorenylmethyloxycarbonyl method) and tBoc method (t-butyloxycarbonyl method). Alternatively, they may also be synthesized by means of a peptide synthesizer manufactured by Sowa Trading Co. (Advanced ChemTech, USA), Perkin Elmer, Amersham Pharmacia Biotech, Aloka (Protein Technology Instrument, USA), Kurabo (Synthecell-Vega, USA), PerSeptive Japan, Ltd. (PerSeptive, USA), Shimadzu Corporation or the like.

Now, the methods for the preparation of antibodies recognizing the protein of the present invention are described below.

(i) Preparation of a polyclonal antibody

A purified full-length or partial fragment of the protein obtained in the above-described manner, or a peptide having a partial amino acid sequence of the protein of the present invention is used as an antigen. A polyclonal antibody may be prepared by administering this antigen to an animal.

As the animal to which the antigen is administered, there may be used a rabbit, goat, mouse, hamster or the like. The dose of the antigen is preferably in the range of 50 to 100 μ g per animal. When a peptide is used, it desirably used after being covalently bonded to carrier protein such as keyhole limpet haemocyanin or bovine thyroglobulin. The peptide used as an antigen may be synthesized by means of a peptide synthesizer.

After the first administration, the antigen is administered 3 to 10 times at intervals of 1 to 2 weeks. After each administration, blood is collected from the venous plexus of fundus oculi on the 3rd to 7th day, and the reaction of the serum with the antigen used for immunization is confirmed by enzyme immunoassay [Enzyme-Linked Immunosorbent assay (ELISA) (in Japanese), Igaku Shoin, 1976; Antibodies-A Laboratory Manual, Cold Spring Harbor Laboratory (1988)] or the like.

Serum is obtained from a nonhuman mammal whose serum exhibits a sufficient antibody titer against the antigen used for immunization. Then, a polyclonal antibody can be obtained by separating and purifying the serum.

The techniques which can be employed for the purpose of separation and purification include centrifugation, salting-out with 40-50% saturated ammonium sulfate, caprylic acid precipitation [Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory (1988)], chromatography using a DEAE-Sepharose column, anion-exchange column, protein A or G column, or gel filtration column, and the like. These techniques may be used either alone or in combination.

(ii) Preparation of a monoclonal antibody

(a) Preparation of antibody-producing cells

A rat whose serum exhibits a sufficient antibody titer against the partial fragment polypeptide of the protein of the present invention used for immunization is used as a source of antibody-producing cells.

After the antigenic substance is finally administered to the rat exhibiting the aforesaid antibody titer, the spleen is excised on the 3rd to 7th day. The spleen is minced in MEM (manufactured by Nissui Seiyaku Co., Ltd.) and loosened with a pincette. After this suspension is centrifuged at 1,200 rpm for 5 minutes, the supernatant is discarded. The spleen cells in the resulting precipitate fraction are treated with a Tris-ammonium chloride buffer (pH 7.65) for 1-2 minutes to remove erythrocytes, and washed three times with MEM. The spleen cells thus obtained are used as antibody-producing cells.

(b) Preparation of myeloma cells

As the myeloma cells, an established cell line obtained from a mouse or rat is used.

Usable cell lines include, for example, the 8-azaguanine-resistant mouse (BALB/c-derived) myeloma cell line P3-X63Ag8-U1 (hereinafter abbreviated as P3-U1) [Curr. Topics. Microbiol. Immunol., 81, 1(1978); Europ. J. Immunol., 6, 511(1976)], SP2/0-Ag14(SP-2) [Nature, 276, 269(1978)], P3-X63-Ag8653(653) [J. Immunol., 123, 1548(1979)], and P3-X63-Ag8(X63) [Nature, 256, 495(1975)].

Such a cell line is subcultured in 8-azaguanine medium [a culture medium prepared by adding glutamine (1.5 mmol/l), 2-mercaptoethanol (5×10^{-5} M), gentamicin (10 μ g/ml) and fetal calf serum (FCS) (manufactured by CSL, 10%) to RPMI-1640 medium (the resulting medium is hereinafter referred to as the normal medium) and further adding 8-azaguanine (15 μ g/ml) thereto]. Three or four days before cell fusion, the cell line is cultured in the normal medium, and not less than 2×10^7 cells are used for the purpose of cell fusion.

(c) Formation of hybridomas

The antibody-producing cells obtained in (a) and the myeloma cells obtained in (b) are thoroughly washed with MEM or PBS (1.83 g disodium phosphate, 0.21 g monopotassium phosphate, 7.65 g sodium chloride, 1 liter distilled water, pH 7.2), and mixed so that the antibody-producing cells and the myeloma cells are present in a ratio of 5-10:1. After this mixture was centrifuged at 1,200 rpm for 5 minutes, the supernatant is discarded.

The mass of cells in the resulting precipitate fraction is thoroughly loosened, and 0.2 to 1 ml (per 10^8 antibody-producing cells) of a solution prepared by mixing 2 g of polyethylene glycol-1000 (PEG 1000), 2 ml of MEM, and 0.7 ml of dimethyl sulfoxide (DMSO) is added to the mass of cells at 37°C with stirring. Moreover, 1 to 2 ml of MEM is added thereto several times at intervals of 1 to 2 minutes. After completion of the addition, MEM is added to make a total volume of 50 ml.

After the suspension so prepared is centrifuged at 900 rpm for 5 minutes, the supernatant is discarded. The cells of the resulting precipitate fraction are gently loosened, and gently suspended in 100 ml of HAT medium [a culture medium prepared by adding hypoxanthine (10^{-4} M), thymidine (1.5×10^{-5} M) and aminopterin (4×10^{-7} M) to the normal medium] by repeated sucking and blowing with a measuring pipette.

This suspension is pipetted into the wells of a 96-well culture plate in an amount of 100 μ l per well, and incubated at 37°C in a 5% CO₂ incubator for 7 to 14 days. After incubation, a portion of the culture supernatant is taken, and hybridomas reacting specifically with the partial fragment polypeptide of the protein of the present invention are selected according to enzyme immunoassay as described in Antibodies, A Laboratory Manual, Cold

Spring Harbor Laboratory, Chapter 14 (1988) or the like.

An exemplary procedure for enzyme immunoassay is described below.

The partial fragment polypeptide of the protein of the present invention, which was used as an antigen at the time of immunization, is coated on a suitable plate and reacted with a first antibody comprising the culture supernatant of a hybridoma or the purified antibody obtained in (d) below, further reacted with a second antibody comprising an anti-rat or anti-mouse immunoglobulin antibody labeled with biotin, an enzyme, a chemiluminescent substance, a radioactive compound or the like, and then subjected to a reaction depending on the labeling material. Thus, the hybridomas reacting specifically with the protein of the present invention are selected as hybridomas for producing a monoclonal antibody against the protein of the present invention.

Using these hybridomas, cloning is repeated twice according to the limiting dilution method [HT medium (HAT medium freed of aminopterin) was used for the first time and the normal medium for the second time]. A hybridoma exhibiting a high antibody titer stably is selected as a hybridoma strain capable of producing an antibody against the polypeptide of the protein of the present invention.

(d) Preparation of a monoclonal antibody

5×10^6 to 20×10^6 cells per animal of the hybridoma capable of producing a monoclonal antibody against the protein of the present invention, which was obtained in (c), is intraperitoneally injected into 8- to 10-weeks-old mice or nude mice having been subjected to a pristane treatment (i.e., having been treated by administering 0.5 ml of 2,6,10,14-tetramethylpentadecane (pristane) intraperitoneally and kept for 2 weeks]. After 10 to 21 days, the hybridoma develops into an ascites tumor. Ascites is collected from a mouse having developed an ascites tumor, and centrifuged at 3,000 rpm for 5 minutes to remove any solid matter. From the resulting supernatant, a monoclonal antibody may be purified and harvested in the same manner as described above in connection with a polyclonal antibody.

The subclass of the antibody may be determined by means of a mouse monoclonal antibody typing kit or a rat monoclonal antibody typing kit. The amount of protein may be

determined by the Lowry method or calculated from the absorbance at 280 nm.

Now, the method for preparing a recombinant virus vector useful for producing the protein of the present invention in a specific human tissue is described below.

The cDNA of the shear stress-responsive gene, which was obtained in the above-described manner, encodes a protein having an activity involved in the formation of an arteriosclerotic lesion.

If necessary, a DNA fragment of appropriate length containing a portion encoding the protein is prepared from the full-length cDNA.

A recombinant virus vector is created by inserting the DNA fragment or the full-length cDNA into a virus vector on the downstream side of a promoter.

This recombinant virus vector is introduced into a packaging cell suited to the vector.

As the packaging cell, there may be used any cell that, when the recombinant virus vector lacks any of the genes encoding proteins necessary for the packaging of the virus, can supply the deficient proteins. For example, there may be used human kidney-derived HEK293 cell or mouse fibroblast cell NIH3T3. The proteins supplied by the packaging cell include mouse retrovirus-derived proteins such as gag, pol and env for a retrovirus vector; HIV virus-derived proteins such as gag, pol, env, vpr, vpu, vif, tat, rev and nef for a lentivirus vector; adenovirus-derived proteins such as E1A and E1B for an adenovirus vector; and proteins such as Rep(p5, p19, p40) and Vp(Cap) for an adeno-associated virus.

As the virus vector, there is used a virus vector which can produce the recombinant virus in the aforesaid packaging cell and which contains a promoter at a position permitting the shear stress-responsive DNA to be transcribed in a target cell. As the plasmid vector, there may be used MFG [Proc. Natl. Acad. Sci. USA, 92, 6733-6737 (1995)], pBabePuro [Nucleic Acids Res., 18, 3587-3596(1990)], LL-CG, CL-CG, CS-CG, CLG [Journal of Virology, 72, 8150-8157(1998)] and pAdex1 [Nucleic Acids Res., 23, 3816-3821(1995)]. The promoter may be any promoter that can be expressed in human tissues. Examples thereof include the promoter of the IE (immediate early) gene of cytomegalovirus (human CMV), the early promoter of SV40, the promoter of retroviruses, metallothionein promoter, heat shock

protein promoter, and SR α promoter. The enhancer of the IE gene of human CMV may be used in conjunction with the promoter.

Examples of the method for introducing the aforesaid recombinant virus vector into the aforesaid packaging cell include the calcium phosphate method (Japanese Published Unexamined Patent Application No. 227075/90) and the lipofection method [Proc. Natl. Acad. Sci. USA, 84, 7413(1987)].

Now, the method for detecting a shear stress-responsive mRNA using the shear stress-responsive DNA of the present invention is described below.

The DNAs which can be used in this method include, for example, DNAs having the nucleotide sequences represented by SEQ ID NO:1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 116, 117, 119, 121, 123, 125, 127, 129, 130, 131, 132, 133, 134, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, 155, 157, 168, 170, 172 and the like. Moreover, they include a DNA having a sequence of part of the foregoing DNA, an oligonucleotide DNA having the nucleotide sequence of 5 to 60 consecutive bases in the foregoing DNA, and preferably an oligonucleotide DNA having the nucleotide sequence of 10 to 40 consecutive bases therein. Furthermore, they include a shear stress-responsive DNA capable of hybridizing with the foregoing DNA or a fragment thereof under stringent conditions.

The identification of a change in the expression level of a shear stress-responsive mRNA and a structural change of the expressed mRNA in human biological specimens and human primary cultured cells is useful in knowing the risk of developing arteriosclerosis in the future or the cause of an already developed vascular disease.

Examples of the method for detecting the expression level of a shear stress-responsive mRNA and a structural change thereof include (1) Northern blotting, (2) in situ hybridization, (3) quantitative PCR, (4) differential hybridization, (5) the DNA chip method, and (6) RNase protection assay.

The materials which can be analyzed by the aforesaid methods include mRNA or total

RNA which is obtained from biological specimens (e.g., vascular endothelium, blood serum and saliva) collected from arteriosclerotic patients and healthy subjects, or a primary cultured cell sample prepared by isolating cells from such a biological specimen and culturing them in a suitable culture medium in vitro (the mRNA and total RNA are hereinafter referred to as the specimen-derived RNA). Alternatively, isolated paraffin or cryostat sections of tissues obtained from biological specimens may also be used.

Northern blotting is a technique in which the specimen-derived RNA is separated by gel electrophoresis, transferred to a support such as a nylon filter, hybridized with a labeled probe prepared from the DNA of the present invention, and then washed to detect a band bound specifically to a shear stress-responsive mRNA. Thus, the expression level of a shear stress-responsive mRNA and a structural change thereof can be detected. The hybridization is carried out by incubating the support under such conditions as a stable hybrid is formed between the probe and a shear stress-responsive mRNA in the specimen-derived RNA. In order to prevent a false positive reaction, it is desirable to carry out the hybridization and washing steps under highly stringent conditions. They are determined according to a large number of factors such as temperature, ionic strength, base composition, probe length and formamide concentration. These factors are described, for example, in *Molecular Cloning*, Second Edition (as mentioned above).

The labeled probe used for Northern blotting may be prepared, for example, by incorporating a radioactive isotope, biotin, a fluorescent group, a chemiluminescent group or the like into the DNA of the present invention or an oligonucleotide designed from the sequence of the DNA, according to a well-known technique (nick translation, random priming or kinasing). Since the amount of labeled probe hybridized reflects the expression level of the shear stress-responsive mRNA, the expression level of the shear stress-responsive mRNA can be determined by determining the amount of labeled probe hybridized. Moreover, a structural change of the shear stress-responsive mRNA can be detected by analyzing the binding site of the labeled probe.

The expression level of a shear stress-responsive mRNA can also be detected by in

situ hybridization in which the hybridization and washing steps are carried out by using the aforesaid labeled probe and isolated paraffin or cryostat sections of tissues obtained from the living body. In order to prevent a false positive reaction in in situ hybridization, it is desirable to carry out the hybridization and washing steps under highly stringent conditions. They are determined according to a large number of factors such as temperature, ionic strength, base composition, probe length and formamide concentration. These factors are described, for example, in Current Protocols in Molecular Biology.

Some methods for detecting a shear stress-responsive mRNA, such as quantitative PCR, differential hybridization, and the DNA chip method, may be carried out on the basis of the synthesis of cDNA by using the specimen-derived RNA, an oligo-dT primer or random primer, and reverse transcriptase (the resulting cDNA is hereinafter referred to as the specimen-derived cDNA). When the specimen-derived RNA is mRNA, both of the aforesaid primers may be used. However, when the specimen-derived RNA is total RNA, it is necessary to use an oligo-dT primer.

In quantitative PCR, DNA fragments derived from the shear stress-responsive mRNA are amplified by carrying out PCR while using a template comprising the specimen-derived cDNA and primers designed on the basis of the nucleotide sequence possessed by the DNA of the present invention. Since the amount of the amplified DNA fragments reflects the expression level of the shear stress-responsive mRNA, the amount of the shear stress-responsive mRNA can be determined by using, as an internal control, a DNA encoding actin or G3PDH (glyceraldehyde 3-phosphate dehydrogenase) not responding to a shear stress. Moreover, a structural change of the shear stress-responsive mRNA can be detected by separating the amplified DNA fragments by gel electrophoresis. In this detection method, it is desirable to use suitable primers capable of amplifying a target sequence specifically and efficiently. Such suitable primers can be designed on the basis of the conditions that they do not hybridize between primers or within primers and that they hybridize specifically with the target cDNA at the annealing temperature and separate from the target under denaturing conditions. The quantitative determination of the amplified DNA fragments must be carried

out within the range of the number of cycles of PCR in which the amplification product is increasing exponentially. Such a number of cycles of PCR can be known by recovering the amplified DNA fragment produced at each number of cycles of PCR and analyzing it by gel electrophoresis.

An alteration of the expression level of the shear stress-responsive mRNA can be detected by hybridizing and washing the DNA of the present invention immobilized on a filter or a substrate (e.g., slide glass or silicon) while using a probe comprising the specimen-derived cDNA synthesized from the specimen-derived RNA with the aid of dNTP. The methods based on this principle include methods called differential hybridization [Trends in Genetics, 7, 314-317(1991)] and the DNA chip method [Genome Research, 6, 639-645(1996)]. In both methods, the difference in the expression of the shear stress-responsive mRNA between a control specimen and a target specimen can be accurately detected by immobilizing an internal control (e.g., actin or G3PDH) on the filter or substrate. Moreover, the accurate expression level of the shear stress-responsive mRNA can be determined by synthesizing cDNAs from a control specimen and the specimen-derived RNA using different labeled dNTP and carrying out hybridization with the two labeled cDNA probes simultaneously on one filter or one substrate.

In RNase protection assay, a promoter sequence (e.g., T7 promoter or SP6 promoter) is first linked to the 3'-terminus of the DNA of the present invention. Then, in an in vitro transcription system using RNA polymerase, a labeled antisense RNA is synthesized using labeled rNTP. After this labeled antisense RNA is combined with the specimen-derived RNA to form an RNA-RNA hybrid, it is digested with RNase and a band protected from digestion is detected by gel electrophoresis. The expression level of the shear stress-responsive mRNA can be determined by assaying the protected band.

Now, the method for detecting a gene causative of arteriosclerosis using the shear stress-responsive DNA of the present invention is described below.

The DNAs which can be used in this method include, for example, DNAs having the nucleotide sequences represented by SEQ ID NO:1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25,

27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 116, 117, 119, 121, 123, 125, 127, 129, 130, 131, 132, 133, 134, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, 155, 157, 168, 170, 172 and the like. Moreover, they include a DNA having a sequence of part of the foregoing DNA, an oligonucleotide DNA having the nucleotide sequence of 5 to 60 consecutive bases in the foregoing DNA, and preferably an oligonucleotide DNA having the nucleotide sequence of 10 to 40 consecutive bases therein. Furthermore, they include a shear stress-responsive DNA capable of hybridizing with the foregoing DNA or a fragment thereof under stringent conditions.

The most accurate test for evaluating the presence or absence of a mutation causative of arteriosclerosis in the locus of a shear stress-responsive gene is a direct comparison of the genes from a control population with the genes from arteriosclerotic patients.

Specifically, human biological specimens such as vascular endothelium, blood serum or saliva, or specimens derived from primary cultured cells established from the biological specimens, are collected from 10 to 100 arteriosclerotic patients and healthy subjects. Then, DNA is extracted from each of the biological specimens or the primary cultured cell-derived specimens (this DNA is hereinafter referred to as the specimen-derived DNA). This specimen-derived DNA may be used directly, or may be used by amplifying a shear stress-responsive DNA using primers designed on the basis of the nucleotide sequence possessed by the DNA of the present invention. Alternatively, PCR may be carried out by using a template comprising the specimen-derived cDNA and primers designed on the basis of the nucleotide sequence possessed by the DNA of the present invention. Thus, a DNA fragment comprising a shear stress-responsive DNA sequence can be amplified and used.

In order to determine whether the DNA of the present invention has a mutation causative of arteriosclerosis, a method for detecting a heteroduplex formed by hybridization between a DNA strand having a wild type allele and a DNA strand having a mutated allele can be used.

The methods which can be used to detect a heteroduplex include (1) the detection of a

heteroduplex by polyacrylamide electrophoresis [Trends Genet., 7, 5(1991)], (2) single strand conformation polymorphism analysis [Genomics, 16, 325-332(1993)], (3) chemical cleavage of mismatches (CCM), (4) enzymatic cleavage of mismatches [Nature Genetics, 9, 103-104(1996)], (5) denaturing gradient gel electrophoresis [Mutat. Res., 288, 103-112(1993)], and the like.

Using a template comprising the specimen-derived DNA or specimen-derived cDNA and primers designed on the basis of the sequence possessed by the DNA of the present invention, a shear stress-responsive DNA is amplified as a fragment smaller than 200 bp, and then subjected to polyacrylamide electrophoresis. If a heteroduplex is formed owing to a mutation of the shear stress-responsive DNA, it has lower mobility than a homoduplex having no mutation, and can hence be detected as extra bands. The use of a specially made gel (Hydro-link, MDE or the like) provides a higher degree of separation. The analysis of a fragment smaller than 200 bp makes it possible to detect an insertion, a deletion, and most one-base substitutions. It is desirable to carry out this heteroduplex analysis on one sheet of gel in combination with single strand conformation polymorphism analysis as described below.

In single strand conformation polymorphism analysis (SSCP analysis), a shear stress-responsive DNA is amplified as a fragment smaller than 200 bp by using a template comprising the specimen-derived DNA or specimen-derived cDNA and primers designed on the basis of the sequence possessed by the DNA of the present invention. The amplified shear stress-responsive DNA fragment is denatured and then electrophoresed through native polyacrylamide gel. During DNA amplification, the primers are labeled with an isotope or fluorochrome, or the unlabeled amplification product is stained with silver. Thus, the amplified shear stress-responsive DNA fragment can be detected as bands. In order to clarify the difference from a wild type pattern, a control specimen may be electrophoresed at the same time. Thus, fragments having a mutation can be detected owing to their difference in mobility.

In chemical cleavage of mismatches (CCM), the shear stress-responsive DNA is

amplified by using a template comprising the specimen-derived DNA or specimen-derived cDNA and primers designed on the basis of the sequence possessed by the DNA of the present invention. The amplified DNA fragment is hybridized with a labeled DNA prepared by incorporating an isotope or fluorescent label into the DNA of the present invention, and treated with osmium tetroxide to cleave one strand of the DNA at a mismatching site. Thus, a mutation can be detected. CCM is one of the most sensitive detection methods and can be applied even to specimens of kilobase length.

In place of the aforesaid osmium tetroxide, a combination of an enzyme involved in the repair of mismatches in cells (e.g., T4 phage resolvase or endonuclease VII) and RNase A may be used. Thus, a mismatch can be cleaved enzymatically.

In denaturing gradient gel electrophoresis (DGGE), the shear stress-responsive DNA is amplified by using a template comprising the specimen-derived DNA or specimen-derived cDNA and primers designed on the basis of the sequence possessed by the DNA of the present invention. The amplified DNA fragment is electrophoresed through a gel having a concentration gradient of a chemical denaturing agent or a temperature gradient. The amplified DNA fragment moves through the gel up to a position where it is denatured into single strands, and cease to move after denaturation. Since the amplified DNA fragments move through the gel differently according to the presence or absence of a mutation in the shear stress-responsive DNA, the presence of a mutation can be detected. In order to enhance detection sensitivity, a poly(G:C) terminus may be attached to each primer.

Another method for determining whether the DNA of the present invention has a mutation causative of arteriosclerosis is a protein truncation test (PTT) [Genomics, 20, 1-4(1994)]. This test can specifically detect a frame shift mutation, splice site mutation or nonsense mutation which develops a deletion in protein. In PTT, a special primer is designed by linking a T7 promoter sequence and a eucaryotic translation initiation sequence to the 5'-terminus of the DNA of the present invention. Using this primer, cDNA is prepared from specimen-derived RNA according to the reverse-transcribed PCR (RT-PCR) technique. When this cDNA is reacted in an in vitro transcription/translation system, it is transcribed into

mRNA by the action of T7 promoter and translated by the action of the translation initiation sequence, so that a protein is produced. When this protein is electrophoresed through a gel, there will be no mutation that develops a deletion if the position of the migrated protein corresponds to that of the full-length protein. If the protein has a deletion, it will be migrated over a shorter distance than the full-length protein. Thus, the degree of deletion can be estimated from that position.

In order to determine the nucleotide sequences of the specimen-derived DNA and the specimen-derived cDNA, it is possible to use primers designed on the basis of the nucleotide sequence of the DNA of the present invention. An analysis of the determined nucleotide sequences makes it possible to judge whether or not the specimen-derived DNA or the specimen-derived cDNA has a mutation causative of arteriosclerosis.

A mutation outside the coding region of a shear stress-responsive gene can be detected by testing non-coding regions such as introns and control sequences near or within the gene. An arteriosclerotic disease caused by a mutation in a non-coding region can be ascertained by comparing the test specimen with a control specimen according to the above-described method and detecting an abnormal size, or abnormal production, of mRNA in the arteriosclerotic patient.

For the gene suggesting the presence of a mutation in a non-coding region, the DNA of the non-coding region can be cloned by using the DNA of the present invention as a probe for hybridization. A mutation in a non-coding region may be searched according to any of the above-described methods.

By subjecting to a statistical analysis according to the method described in Handbook of Human Genetics Linkage, The John Hopkins University Press, Baltimore (1994), the mutations so found may be identified as single nucleotide polymorphisms (SNPs) having a linkage with arteriosclerosis. Moreover, a gene causative of arteriosclerosis may be identified by obtaining DNA from a family having a history of arteriosclerosis according to the previously described method, and detecting a mutation therefrom.

Now, the method for diagnosing vascular diseases caused by arteriosclerosis using the

shear stress-responsive DNA of the present invention is described below.

The DNAs which can be used in this method include, for example, DNAs having the nucleotide sequences represented by SEQ ID NO:1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 116, 117, 119, 121, 123, 125, 127, 129, 130, 131, 132, 133, 134, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, 155, 157, 168, 170, 172 and the like. Moreover, they include a DNA having a sequence of part of the foregoing DNA, an oligonucleotide DNA having the nucleotide sequence of 5 to 60 consecutive bases in the foregoing DNA, and preferably an oligonucleotide DNA having the nucleotide sequence of 10 to 40 consecutive bases therein. Furthermore, they include a shear stress-responsive DNA capable of hybridizing with the foregoing DNA or a fragment thereof under stringent conditions.

The cause of arteriosclerosis can be ascertained by detecting a gene mutation in any tissue of a human subject. For example, where a mutation is present in the germ cell system, an individual having inherited the mutation may tend to develop arteriosclerosis. The mutation can be identified by testing DNA obtained from any tissue of the body of the individual. For example, a diagnosis of arteriosclerosis can be made by collecting blood from a subject, extracting DNA from cells of the blood, and using this DNA to perform a test for a gene mutation. Moreover, a prenatal diagnosis may be made by using fetal cells, placental cells or amniotic cells to perform a test for a gene mutation.

Furthermore, by obtaining a biological tissue from the lesion of a patient having developed a vascular disease and testing its DNA, the type of the vascular disease can be diagnosed and the results thus obtained can be utilized to select a drug to be administered. In order to detect a mutation of a gene in the tissue, it is useful to isolate the lesional tissue segregated from the surrounding normal tissues. An arteriosclerotic lesion may be obtained, for example, by a bypass operation for replacing the lesion of arteriosclerosis with a normal blood vessel. The tissue thus obtained is treated with trypsin or the like, and the resulting cells are cultured in a suitable culture medium. Then, chromosomal DNA and mRNA can be

extracted from the cultured cells.

The DNA obtained from a human specimen for diagnostic purposes according to any of the aforesaid methods is hereinafter referred to as the diagnostic specimen-derived DNA. Moreover, the cDNA synthesized from the RNA obtained from a human specimen for diagnostic purposes according to any of the aforesaid methods is hereinafter referred to as the diagnostic specimen-derived cDNA.

Using the shear stress-responsive DNA of the present invention and the diagnostic specimen-derived DNA or the diagnostic specimen-derived cDNA, a diagnosis of arteriosclerosis may be made according to the above-described method for detecting a gene causative of arteriosclerosis.

Moreover, in order to make a diagnosis of arteriosclerosis by using the shear stress-responsive DNA of the present invention and the diagnostic specimen-derived DNA or the diagnostic specimen-derived cDNA, there may also be employed a method such as (1) the detection of a restriction enzyme site, (2) the utilization of an allele-specific oligonucleotide probe [allele-specific oligonucleotide hybridization (ASO)], (3) PCR using an allele-specific oligonucleotide [amplification refractory mutation system (ARMS)], (4) oligonucleotide ligation assay (OLA), (5) PCR-preferential homoduplex formation assay (PCR-PHFA), or (6) oligo-DNA array [Protein-Nucleic Acid-Enzyme (in Japanese), 43, 2004-2011(1998)].

Where a restriction enzyme site disappears or appears as a result of a single base change, the mutation may be easily detected by amplifying the diagnostic specimen-derived DNA or the diagnostic specimen-derived cDNA with primers designed on the basis of the sequence possessed by the DNA of the present invention, digesting it with the restriction enzyme, and comparing the resulting restriction enzyme-cleaved DNA fragments with those obtained from healthy subjects. However, the occurrence of such a mutation is rare. For diagnostic purposes, a mismatch exerting no influence on annealing is introduced into PCR primers designed on the basis of the sequence possessed by the DNA of the present invention. Thus, for a mutation not accompanied by the disappearance or appearance of a restriction enzyme site, a restriction enzyme site is artificially introduced.

A short synthetic DNA probe hybridizes only with a perfectly base pairing sequence alone. Taking advantage of this characteristic, a single-base mutation can be easily detected by preparing an allele-specific oligonucleotide probe (ASO). For diagnostic purposes, reverse dot blotting is often employed in which an oligonucleotide designed on the basis of the sequence possessed by the DNA of the present invention and an identified mutation is attached to a filter and hybridization is carried out with a probe prepared from the diagnostic specimen-derived DNA or the diagnostic specimen-derived cDNA by PCR using primers designed on the basis of the sequence possessed by the DNA of the present invention and labeled dNTP. In the DNA chip method, an oligonucleotide designed on the basis of the sequence possessed by the DNA of the present invention and the mutation is synthesized directly on a substrate (e.g., slide glass or silicon) to form a highly dense array. This DNA chip method is a mutation detection method suitable for large-scale diagnostic purposes because various mutations can be more conveniently detected by using a small amount of the diagnostic specimen-derived DNA or the diagnostic specimen-derived cDNA.

Nucleotide mutations can also be detected by the following oligonucleotide ligation assay (OLA).

Two oligonucleotides consisting of about 20 bases, which are designed from the sequence possessed by the DNA of the present invention and are capable of hybridizing on both sides of a mutation site, are prepared. Using a template comprising the diagnostic specimen-derived DNA or the diagnostic specimen-derived cDNA and primers designed from the sequence possessed by the DNA of the present invention, shear stress-responsive DNA fragment is amplified by PCR. The amplified fragment is hybridized with the aforesaid polynucleotide. After hybridization, the two oligonucleotides are ligated by means of DNA ligase. For example, by labeling one oligonucleotide with biotin and the other oligonucleotide with a different label such as digoxigenin, it is possible to detect rapidly whether the ligation has occurred or not. OLA is a mutation detection method suitable for large-scale diagnostic purposes because it does not require electrophoresis or centrifugation.

A very small amount of a mutated gene can also be quantitatively and easily detected

by the following PCR-PHFA.

PCR-PHFA is a combination of three techniques including gene amplification (PCR), liquid-phase hybridization exhibiting very high specificity, and enzymatic detection of PCR product (ED-PCR) detecting the PCR product in the same manner as ELISA. Using a dinitrophenyl(DNP)-labeled and biotin-labeled primer set, an amplification product labeled at both ends is prepared by carrying out PCR amplification while using the DNA of the present invention as a template. This amplification product is mixed with a large excess (20- to 100-fold) of an unlabeled amplification product obtained by using an unlabeled primer set having the same sequences and a template comprising the diagnostic specimen-derived DNA or the diagnostic specimen-derived cDNA. After thermal denaturation, this mixture is cooled with a gentle temperature gradient of the order of 1°C/5-10 minutes to form perfect complementary strands preferentially. The labeled DNA so reformed is captured and adsorbed to a streptavidin-immobilized well via biotin. On the other hand, an enzyme-labeled anti-DNP antibody is bound thereto via DNP. Thus, the labeled DNA can be detected by a color-developing reaction based on the enzyme. If a gene having the same sequence as the labeled DNA is not present in the specimen, the original double-strand labeled DNA is preferentially reformed to develop a color. In contrast, if a gene having the same sequence is present in the specimen, complementary strands are randomly replaced to decrease the reformation of the labeled DNA, resulting in a marked reduction in color development. Thus, known mutated polymorphic genes can be detected and quantitatively determined.

Now, the methods for detection and quantitative determination of the shear stress-responsive protein of the present invention immunologically using the antibody of the present invention are described below.

The methods by which a microorganism, an animal cell or an insect cell, or a tissue, expressing the protein of the present invention intracellularly or extracellularly can be immunologically detected and determined quantitatively using the antibody (polyclonal antibody or monoclonal antibody) of the present invention include fluorescent antibody technique, enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA),

immunohistochemical staining techniques (e.g., ABC method and CSA method) such as tissue immunostaining and cell immunostaining, western blotting, dot blotting, immunoprecipitation, sandwich ELISA [Experimental Manual for Monoclonal Antibodies (in Japanese), Kodansha Scientific (1987); Biochemical Experimental Lectures (Second Series) 5, Methods of Immunobiochemical Research (in Japanese), Tokyo-Kagaku-Dojin (1986)], and the like.

The fluorescent antibody technique is a technique in which, after a microorganism, an animal cell or an insect cell, or a tissue, expressing the protein of the present invention intracellularly or extracellularly is reacted with the antibody of the present invention and further reacted with an anti-mouse IgG antibody, or a fragment thereof, labeled with a fluorescent material such as fluorescein isothiocyanate (FITC), fluorescence is measured with a flow cytometer.

Enzyme-linked immunosorbent assay (ELISA) is a technique in which, after a microorganism, an animal cell or an insect cell expressing the protein of the present invention intracellularly or extracellularly is reacted with the antibody of the present invention and further reacted with an anti-mouse IgG antibody, or a binding fragment thereof, labeled with an enzyme such as peroxidase or biotin, the developed color is measured with a spectrophotometer.

Radioimmunoassay (RIA) is a technique in which, after a microorganism, an animal cell or an insect cell, or a tissue, expressing the protein of the present invention intracellularly or extracellularly is reacted with the antibody of the present invention and further reacted with an anti-mouse IgG antibody, or a fragment thereof, labeled with a radioactive substance, radioactivity is measured with a scintillation counter or the like.

Cell immunostaining and tissue immunostaining are in which, after a microorganism, an animal cell or an insect cell, or a tissue, expressing the protein of the present invention intracellularly or extracellularly is reacted with the antibody of the present invention and further reacted with an anti-mouse IgG antibody, or a fragment thereof, labeled with a fluorescent material (e.g., FITC) or an enzyme (e.g., peroxidase or biotin), it is observed

under the microscope.

Western blotting is a technique in which, after an extract of a microorganism, an animal cell or an insect cell, or a tissue, expressing the protein of the present invention intracellularly or extracellularly is fractionated by SDS-polyacrylamide gel electrophoresis [Antibodies-A Laboratory Manual, Cold Spring Harbor Laboratory (1988)], this gel is blotted to a PVDF membrane or a nitrocellulose membrane, then the membrane is reacted with the antibody of the present invention and further reacted with an anti-mouse IgG antibody, or a fragment thereof, labeled with a fluorescent material (e.g., FITC) or an enzyme (e.g., peroxidase or biotin). Thus, the protein of the present invention can be detected.

Dot blotting is a technique in which, after an extract of a microorganism, an animal cell or an insect cell, or a tissue, expressing the protein of the present invention intracellularly or extracellularly is blotted to a nitrocellulose membrane, this membrane is reacted with the antibody of the present invention and further reacted with an anti-mouse IgG antibody, or a binding fragment thereof, labeled with a fluorescent material (e.g., FITC) or an enzyme (e.g., peroxidase or biotin). Thus, the protein of the present invention can be detected.

Immunoprecipitation is a technique in which, after an extract of a microorganism, an animal cell or an insect cell, or a tissue, expressing the protein of the present invention intracellularly or extracellularly is extracted, the resulting extract is reacted with the antibody of the present invention, a carrier having the ability to bind specifically to immunoglobulin (e.g., protein G-Sepharose) is added thereto so as to precipitate the resulting antigen-antibody complex.

In sandwich ELISA, two antibodies of the present invention having different antigen recognition sites are provided. In advance, one of the antibodies is adsorbed to a plate, and the other is labeled with a fluorescent material (e.g., FITC) or an enzyme (e.g., peroxidase or biotin). After an extract of a microorganism, an animal cell or an insect cell, or a tissue, expressing the protein of the present invention intracellularly or extracellularly is extracted, the resulting extract is reacted with the antibody-adsorbed plate, the plate is reacted with the labeled antibody and subjected to a reaction depending on the labeling material.

Now, the method for diagnosing vascular diseases caused by arteriosclerosis using the antibody of the present invention is described below.

The identification of an alteration of the expression level of a shear stress-responsive protein and a structural change of the expressed protein in human biological specimens and human primary cultured cells is useful in knowing the risk of developing arteriosclerosis in the future or the cause of an already developed vascular disease.

The methods which can be employed to make a diagnosis by detecting the expression level of a shear stress-responsive protein and a structural change thereof include the above-described fluorescent antibody technique, enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), immunohistochemical staining techniques (e.g., ABC method and CSA method) such as tissue immunostaining and cell immunostaining, western blotting, dot blotting, immunoprecipitation, sandwich ELISA and the like.

The materials which can be diagnosed by the aforesaid methods include biological specimens themselves (e.g., blood vessels in the lesion, blood, serum, urine, feces and saliva) collected from human subjects, and cells or cell extracts obtained from the foregoing biological specimens. Alternatively, an isolated paraffin or cryostat section of a tissue obtained from biological specimens may also be used.

Now, the methods for screening a therapeutic agent for vascular diseases caused by arteriosclerosis using the shear stress-responsive DNA of the present invention, a protein encoded by the DNA, or an antibody capable of recognizing the protein are described below.

The DNAs which can be used in these screening methods include, for example, DNAs having the nucleotide sequences represented by SEQ ID NO:1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 116, 117, 119, 121, 123, 125, 127, 129, 130, 131, 132, 133, 134, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, 155, 157, 168, 170, 172 and the like; and a shear stress-responsive DNA capable of hybridizing with the foregoing DNA or a fragment thereof under stringent conditions. The protein which can be used therein include a protein encoded by the foregoing

DNA (e.g., a protein having an amino acid sequence selected from the amino acid sequences represented by SEQ ID NO:144, 146, 148, 150, 152, 154, 156, 158, 169, 171 and 173); and a protein comprising an amino acid sequence in which one or more amino acids are deleted, replaced or added as compared with the amino acid sequence possessed by the foregoing protein, and having an activity involved in the formation of an arteriosclerotic lesion. The antibody which can be used therein include an antibody capable of recognizing the foregoing protein.

A microorganism, an animal cell or an insect cell transformed by introducing the DNA of the present invention so as to produce the protein of the present invention or a partial polypeptide of the protein, and the protein or polypeptide in purified form, are useful for the purpose of screening an agent acting specifically on a shear stress-responsive protein. The agent obtained by this screening is useful for the treatment of vascular diseases caused by arteriosclerosis.

One of the aforesaid screening methods comprises selecting a labeled compound binding specifically to a microorganism, an animal cell or an insect cell transformed so as to produce the protein of the present invention or a partial polypeptide of the protein (hereinafter referred to as the transformant for screening). The specific binding of a labeled compound may be detected by comparison with a control comprising an untransformed microorganism, animal cell or insect cell. Alternatively, an unlabeled compound may be selected by competitive screening in which its inhibitory effect on the binding to the transformant for screening of a compound or protein binding specifically to the transformant for screening is used as an index.

The purified protein of the present invention or the purified partial polypeptide of the protein may be used to select a labeled compound binding specifically to a shear stress-responsive protein. The binding of the labeled compound may be determined quantitatively according to the above-described immunological method using the antibody of the present invention. Alternatively, an unlabeled compound may be selected by competitive screening

in which its inhibitory effect on the binding to the protein or polypeptide of a labeled compound binding to the protein or polypeptide is used as an index.

In the other of the aforesaid screening methods, a large number of partial peptides of the protein are densely synthesized on plastic pins or a certain solid support. Thus, a compound or protein binding selectively to the peptides can be efficiently screened (WO 84/03564).

An expression regulating agent capable of regulating the expression of a shear stress-responsive mRNA or protein in vascular endothelial cells is also useful for the treatment of vascular diseases caused by arteriosclerosis.

An agent for regulating the transcription or translation of a shear stress-responsive gene can be screened by adding various compounds to a vascular endothelial cell line and assaying an increase or decrease in the expression of a shear stress-responsive mRNA using the DNA of the present invention. An increase or decrease in the expression of a shear stress-responsive mRNA may be detected by the above-described PCR, Northern blotting, and RNase protection assay.

An agent for regulating the transcription or translation of a shear stress-responsive gene can also be screened by adding various compounds to a vascular endothelial cell line and assaying an increase or decrease in the expression of a shear stress-responsive protein using the antibody of the present invention. An increase or decrease in the expression of a shear stress-responsive protein may be detected by the above-described fluorescent antibody technique, enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), immunohistochemical staining techniques (e.g., ABC method and CSA method) such as tissue immunostaining and cell immunostaining, western blotting, dot blotting, immunoprecipitation, and sandwich ELISA.

The compound obtained by the aforesaid methods may be administered, as an agent, to model animals for arteriosclerosis, such as ApoE knockout mice and rabbits fed with a high cholesterol diet. By measuring the incorporation of oxidized LDL and cholesterol into the vascular endothelium and the formation of an arteriosclerotic lesion in these animals, the

therapeutic effect of the compound on the vascular disease caused by arteriosclerosis can be evaluated.

Now, the drug delivery method using the antibody of the present invention is described below.

The antibody used for this drug delivery may be any antibody in accordance with the present invention. However, it is particularly desirable to use a humanized antibody.

Usable humanized antibodies include a human chimeric antibody, a complementary determining region (hereinafter referred to as CDR) grafted humanized antibody, and the like.

The human chimeric antibody means an antibody consisting of a heavy-chain variable region (the heavy chain may hereinafter be referred to as H chain, the variable region as V region, and the heavy-chain variable region as HV or VH) and a light-chain variable region (the light chain may hereinafter be referred to as L chain, and the light-chain variable region as LV or VL) of an antibody of an animal other than human, and a heavy-chain constant region (the constant region may hereinafter be referred to as C region, and the heavy-chain constant region as CH) and a light-chain constant region (the light-chain constant region may hereinafter be referred to as CL) of a human antibody. As the animal other than human, there may be used any of various animals that permit the generation of hybridomas, such as mice, rats, hamsters and rabbits.

The human chimeric antibody of the present invention may be produced by obtaining cDNAs encoding VH and VL from a hybridoma capable of producing a monoclonal antibody which binds to the protein of the present invention and neutralizes the action of the protein of the present invention; inserting them into an expression vector for mammalian cells having genes encoding human antibody CH and human antibody CL, respectively, to construct a human chimeric antibody expression vector; and introducing the vector into mammalian cells to express the antibody.

The CH of the human chimeric antibody may be any CH belonging to human immunoglobulin (hereinafter abbreviated as hIg). However, the CH of hIgG class is preferred. Furthermore, there may be used any of various subclasses (e.g., hIgG1, hIgG2, hIgG3 and

hIgG4) belonging to hIgG class. The CL of the human chimeric antibody may be any CL belonging to hIg, and the CL of κ or λ class may be used.

The CDR-grafted humanized antibody means an antibody in which the amino acid sequences of CDRs of VH and VL of an antibody of an animal other than human are transplanted into appropriate positions of VH and VL of a human antibody.

The CDR-grafted humanized antibody of the present invention may be produced by constructing cDNAs encoding V regions in which the CDR sequences of VH and VL of any human antibody are replaced by the CDR sequences of VH and VL, respectively, of an antibody of an animal other than human that reacts with the protein of the present invention, binds to the protein of the present invention, and neutralizes the action of the protein of the present invention; inserting them into an expression vector for mammalian cells having genes encoding human antibody CH and human antibody CL, respectively, to construct a CDR-grafted humanized antibody expression vector; and introducing the vector into animal cells to express the antibody.

The CH of the CDR-grafted humanized antibody may be any CH belonging to hIg. However, the CH of hIgG class is preferred. Furthermore, there may be used any of various subclasses (e.g., hIgG1, hIgG2, hIgG3 and hIgG4) belonging to hIgG class. The CL of the CDR-grafted humanized antibody may be any CL belonging to hIg, and the CL of κ or λ class may be used.

Originally, human antibodies mean antibodies existing naturally in the human body. However, they also include antibodies obtained from a human antibody phage library and a human antibody-producing transgenic animal which have been created on the basis of the recent progress of genetic engineering, cell technology and embryological engineering.

An antibody existing in the human body may be obtained, for example, according to the following method.

Lymphocytes are isolated from human peripheral blood, immortalized by infection with EB virus or the like, and then cloned. After the selected lymphocyte producing a desired antibody is cultured, the antibody can be obtained from the resulting culture.

The human antibody phage library is a library in which an antibody gene prepared from human B cells is inserted into a phage gene so as to express antibody fragments (e.g., Fab and single-chain antibody) on the phage surface. From this library, a phage expressing an antibody fragment having a desired antigen-binding activity can be recovered by using its binding activity for a substrate having the antigen immobilized thereon as an index. This antibody fragment can further be converted into a complete human antibody according to genetic engineering techniques.

The human antibody-producing transgenic animal means an animal in which a human antibody gene is introduced into the cells. Specifically, a human antibody-producing transgenic animal may be created by introducing a human antibody gene into a mouse ES cell, transplanting the ES cell into an early embryo of another mouse, and developing the embryo. In order to prepare a human antibody from the human antibody-producing transgenic animal, there may be employed a method which comprises obtaining a human antibody-producing hybridoma according to the common method for the formation of hybridomas in mammals other than human, and culturing the hybridoma to produce and accumulate the human antibody in the resulting culture.

The antibody fragments include Fab, Fab', F(ab')₂, single-chain antibody, dsFv, CDR-containing peptides, and the like.

Among the fragments obtained by treating IgG with the proteolytic enzyme papain (IgG is cleaved at the 224th amino acid residue of each H chain), Fab is an antibody fragment with a molecular weight of about 50,000 which has an antigen-binding activity and consists of about a half of an H chain on the N-terminal side and a whole L chain which are linked together via a disulfide bond.

The Fab of the present invention may be obtained from an antibody reacting specifically with the protein of the present invention, by treating it with the proteolytic enzyme papain. Alternatively, Fab may also be obtained by inserting DNA encoding the Fab of the antibody into a procaryotic expression vector or a eucaryotic expression vector, and introducing the resulting vector into a procaryote or eucaryote to express the DNA.

Among the fragments obtained by treating IgG with the proteolytic enzyme pepsin (IgG is cleaved at the 234th amino acid residue of each H chain), $F(ab')_2$ is an antibody fragment with a molecular weight of about 100,000 which has an antigen-binding activity and is slightly larger than two Fab molecules linked together via disulfide bonds in the hinge area.

The $F(ab')_2$ of the present invention may be obtained from an antibody reacting specifically with the protein of the present invention, by treating it with the proteolytic enzyme pepsin. Alternatively, $F(ab')_2$ may also be obtained by inserting DNA encoding the $F(ab')_2$ of the antibody into a procaryotic expression vector or an eucaryotic expression vector, and introducing the resulting vector into a procaryote or eucaryote to express the DNA.

Fab' is an antibody fragment with a molecular weight of about 50,000 which has an antigen-binding activity and is obtained by breaking the disulfide bonds in the hinge area of the aforesaid $F(ab')_2$.

The Fab' of the present invention may be obtained from an antibody reacting specifically with the protein of the present invention, by treating it with the reducing agent dithiothreitol. Alternatively, Fab' may also be obtained by inserting DNA encoding the Fab' fragment of the antibody into a procaryotic expression vector or an eucaryotic expression vector, and introducing the resulting vector into a procaryote or eucaryote to express the DNA.

A single-chain antibody (hereinafter also referred to as scFv) is a VH-P-VL or VL-P-VH polypeptide consisting of one VH and one VL linked together by a suitable peptide linker (hereinafter referred to as P). The VH and VL contained in the scFv used in the present invention may be those of any antibody (e.g., humanized antibody or human antibody) reacting specifically with the protein of the present invention.

The single-chain antibody of the present invention may be obtained according to the following method.

After cDNAs encoding the VH and VL of an antibody reacting specifically with the protein of the present invention are obtained, a DNA encoding the single-chain antibody is constructed. Then, the single-chain antibody may be obtained by inserting the DNA into a

procaryotic expression vector or an eucaryotic expression vector, and introducing the resulting vector into a procaryote or eucaryote to express the DNA.

The disulfide-stabilized V region fragment (hereinafter referred to as dsFv) is a fragment obtained by replacing one amino acid residue of each of VH and VL with a cysteine residue and linking these polypeptides together by a disulfide bond extending between the cysteine residues. The amino acid residues to be replaced with cysteine residues can be selected on the basis of the predicted stereostructure of the antibody, according to the method shown by Reiter et al. [Protein Engineering, 7, 697(1994)]. The VH and VL contained in the disulfide-stabilized V region fragment used in the present invention may be those of any antibody (e.g., humanized antibody or human antibody) reacting specifically with the protein of the present invention.

The disulfide-stabilized V region fragment of the present invention may be obtained according to the following method.

After cDNAs encoding the VH and VL of an antibody reacting specifically with the protein of the present invention are obtained, a DNA encoding the disulfide-stabilized V region fragment is constructed. Then, the disulfide-stabilized V region fragment may be obtained by inserting the DNA into a procaryotic expression vector or an eucaryotic expression vector, and introducing the resulting vector into a procaryote or eucaryote to express the DNA.

The CDR-containing peptides may be prepared by chemical synthesis processes such as the Fmoc method and the tBoc method.

A conjugated antibody prepared from the antibody of the present invention as described below may be used as a drug delivery system to deliver an agent or protein specifically to a lesion of arteriosclerosis.

The conjugated antibody is an antibody obtained by linking a radioactive isotope, protein, low-molecular-weight agent or the like to an antibody reacting specifically with the protein of the present invention (e.g., a humanized antibody, a human antibody, or a fragment of these antibodies) by chemical means or genetic engineering means.

The conjugated antibody of the present invention may be prepared by linking a radioactive isotope, protein, low-molecular-weight agent or the like to N-terminal or C-terminal side of an H chain or L chain of an antibody or antibody fragment reacting specifically with the protein of the present invention, to a suitable substituent group or side chain of the antibody or antibody fragment, or to a sugar chain of the antibody or antibody fragment, by chemical means or genetic engineering means.

Usable radioactive isotopes include ^{131}I , ^{125}I and the like. They can be linked to an antibody or an antibody fragment, for example, according to the chloramine T method.

Usable low-molecular-weight agents are anticancer drugs including, for example, alkylating agents such as nitrogen mustard and cyclophosphamide; antimetabolites such as 5-fluorouracil and methotrexate; antibiotics such as daunomycin, bleomycin, mitomycin C, daunorubicin and doxorubicin; plant alkaloids such as vincristine, vinblastine and vindesine; and hormones such as tamoxifen and dexamethasone [Clinical Oncology (in Japanese), edited by the Japanese Society for the Research of Clinical Oncology, 1996, Gan-to-Kagakuryoho Sha]; anti-inflammatory drugs including, for example, steroids such as hydrocortisone and prednisone; nonsteroidal anti-inflammatory drugs such as aspirin and indomethacin; immunomodulators such as gold sodium thiomalate and penicillamine; immunosuppressants such as cyclophosphamide and azathioprine; and antihistamines such as chlorpheniramine maleate and clemastine [Inflammation and Anti-inflammatory Therapy (in Japanese), 1982, Ishiyaku Shuppan Kabushiki Kaisha]; and the like.

A low-molecular-weight agent may be linked to the aforesaid antibody in the usual manner. For example, daunomycin may be linked to the antibody, for example, by linking daunomycin to an amino group of the antibody via glutaraldehyde, or by linking the amino group of daunomycin to a carboxyl group of the antibody via water-soluble carbodiimide.

Suitable proteins include cytokines activating immunocompetent cells, and growth controlling factors for vascular endothelium, vascular smooth muscle and the like. Examples thereof include human interleukin 2, human granulocyte macrophage colony-stimulating factor, human macrophage colony-stimulating factor, human interleukin 12, fibroblast growth

factor 2 (FGF-2) and platelet-derived growth factor (PDGF). Moreover, in order to damage directly with proliferative vascular smooth muscle cells of an arteriosclerotic lesion, there may be used a toxin such as ricin or diphtheria toxin.

The conjugated antibody having a protein linked thereto may be prepared according to the following method.

A DNA encoding the conjugated antibody is constructed by linking cDNA encoding the protein to cDNA encoding the antibody or antibody fragment. After this DNA is inserted into a prokaryotic or eucaryotic expression vector, the resulting expression vector is introduced into a procaryote or eucaryote to express the DNA. Thus, the desired conjugated antibody can be obtained.

Now, the method of gene therapy using a virus vector containing the shear stress-responsive DNA of the present invention is described below.

A therapeutic agent may be prepared from the above-described recombinant virus vector and a base for gene therapeutic agents [Nature Genet., 8, 42(1994)].

The base for gene therapeutic agents may be any base that is commonly used for injections. Examples thereof include distilled water; a solution of a salt such as sodium chloride or a mixture of sodium chloride and an inorganic salt; a solution of mannitol, lactose, dextran, glucose or the like; a solution of an amino acid such as glycine or arginine; and a mixture of an organic acid solution or a salt solution and a glucose solution. Moreover, injections in the form of solutions, suspensions or dispersions may be prepared in the usual manner, by using auxiliaries such as osmotic pressure regulators, pH regulators, vegetable oils (e.g., sesame oil and soybean oil) and surfactants (e.g., lecithin and nonionic surfactants), in combination with the aforesaid bases. These injections may also be prepared as preparations to be dissolved at the time of use, according to a technique such as powdering or freeze-drying. Where the gene therapeutic agent of the present invention is a liquid, it may be used directly for therapeutic purposes, as required. Where it is a solid, it may be dissolved immediately before gene therapy in the aforesaid base having been sterilized as required, and used for therapeutic purposes. In order to administer the gene therapeutic agent of the present

invention, there may be employed a local administration method using a double balloon catheter or the like so that the gene therapeutic agent will be absorbed into the vascular endothelium of the treated site of the patient.

As a method for carrying a virus vector more specifically to an arteriosclerotic lesion, Somia et al. have reported a method using a fusion protein consisting of a single-chain antibody capable of recognizing specifically the LDL receptor and the Env protein of a retrovirus vector [Proc. Natl. Acad. Sci. USA, 92, 7570-7574(1995)]. This system is not limited to retrovirus vectors, but may also be applied to lentivirus vectors and the like.

The nonviral gene transfer techniques which are known in this field include calcium phosphate coprecipitation [Virology, 52, 456-467(1973); Science, 209, 1414-1422(1980)], microinjection [Proc. Natl. Acad. Sci. USA, 77, 5399-5403(1980); Proc. Natl. Acad. Sci. USA, 77, 7380-7384(1980); Cell, 27, 223-231(1981); Nature, 294, 92-94(1981)], membrane fusion-mediated transfer method using liposomes [Proc. Natl. Acad. Sci. USA, 84, 7413-7417(1987); Biochemistry, 28, 9508-9514(1989); J. Biol. Chem., 264, 12126-12129(1989); Hum. Gene Ther., 3, 267-275(1992); Science, 249, 1285-1288(1990); Circulation, 83, 2007-2011(1992)], direct DNA incorporation and receptor-mediated DNA transfer method [Science, 247, 1465-1468(1990); J. Biol. Chem., 266, 14338-14342(1991); Proc. Natl. Acad. Sci. USA, 87, 3655-3659(1991); J. Biol. Chem., 264, 16985-16987(1989); BioTechniques, 11, 474-485(1991); Proc. Natl. Acad. Sci. USA, 87, 3410-3414(1990); Proc. Natl. Acad. Sci. USA, 88, 4255-4259(1991); Proc. Natl. Acad. Sci. USA, 87, 4033-4037(1990); Proc. Natl. Acad. Sci. USA, 88, 8850-8854(1991); Hum. Gene Ther., 3, 147-154(1991)], and the like.

When gene transfer using a virus vector is combined with direct in vivo gene transfer using liposome delivery, the virus vector can be directed to an arteriosclerotic lesion.

In addition, a virus vector may also be prepared by combining a DNA of appropriate size in accordance with the present invention with a polylysine-conjugated antibody specific for adenovirus hexon protein to form a complex, and linking the resulting complex to an adenovirus vector. This virus vector attains target cells stably, is incorporated into the cells

by the action of endosomes, and is decomposed in the cells. Thus, the gene can be expressed efficiently.

In an investigation on tumors, it has been reported that membrane fusion-mediated transfer method using liposomes permits a liposome preparation to be administered directly to a target tissue, and the tissue can hence incorporate and express the gene locally [Hum. Gene Ther., 3, 399-410(1992)]. Accordingly, it may be expected that a similar effect is produced in the case of an arteriosclerotic lesion. In order to deliver DNA directly to an arteriosclerotic lesion, it is preferable to employ a gene transfer technique. Receptor-mediated DNA transfer is carried out, for example, by conjugating DNA (usually taking the form of a covalently closed supercoiled plasmid) to a protein ligand via polylysine. The ligand is selected on the basis of the presence of the corresponding ligand receptor on the cell surface of the target cells or tissue. Examples of the combination of the receptor and the ligand include the combination of LDL receptor and LDL, and the combination of scavenger receptor and oxidized LDL. If desired, this ligand-DNA conjugate may be directly injected into the blood and thereby delivered to a target tissue where it binds to the receptor and the DNA-protein complex is internalized. In order to prevent the intracellular degradation of DNA, the target tissue may be simultaneously infected with an adenovirus to disrupt the endosome function.

Now, the method of treatment using an antibody capable of recognizing specifically the shear stress-responsive DNA of the present invention is described below.

A pharmaceutical containing the antibody of the present invention may be administered alone as a therapeutic agent. However, it is usually desirable to provide as pharmaceutical preparations produced by blending the antibody with one or more pharmacologically acceptable carriers and working up the resulting blend according to any of various techniques known well in the technical field of pharmaceuticals.

It is desirable to use the route of administration which is most effective for the purpose of treatment. Examples thereof include oral administration and parenteral administration such as buccal, intratracheal, intrarectal, subcutaneous, intramuscular and intravenous administration. In the case of antibody preparations, intravenous administration is desirable.

Examples of dosage forms include sprays, capsules, tablets, granules, medicated syrups, emulsions, suppositories, injections, ointments, tapes, and the like.

Pharmaceutical preparations suitable for oral administration include emulsions, medicated syrups, capsules, tablets, powders, granules and the like.

Liquid preparations such as emulsions and medicated syrups may be produced using additives including water; sugars such as sucrose, sorbitol and fructose; glycols such as polyethylene glycol and propylene glycol; oils such as sesame oil, olive oil and soybean oil; antiseptics such as p-hydroxybenzoic acid esters; flavors such as strawberry flavor and peppermint; and the like.

Capsules, tablets, powders, granules and the like may be produced using additives including excipients such as lactose, glucose, sucrose and mannitol; disintegrators such as starch and sodium alginate; lubricants such as magnesium stearate and talc; binders such as polyvinyl alcohol, hydroxypropylcellulose and gelatin; surfactants such as fatty acid esters; plasticizers such as glycerin; and the like.

Pharmaceutical preparations suitable for parenteral administration include injection, suppository, spray and the like. Injection is prepared using a carrier comprising a salt solution, a glucose solution or a mixture thereof, and the like. Alternatively, a powder for injection may be prepared by freeze-drying the antibody of the present invention in the usual manner and adding sodium chloride thereto. Suppository is prepared using a carrier such as cacao butter, hydrogenated fats and carboxylic acids.

Spray is prepared by using the antibody of the present invention itself, or using a carrier which does not irritate the mucous membranes of the oral cavity and respiratory tract of the patient and enables the antibody of the present invention to be dispersed as fine particles and easily absorbed, and the like.

Specific examples of the carrier include lactose and glycerin. Depending on the antibody of the present invention and the nature of the carrier used, aerosols, dry powders and the like may be prepared. Also in these parenteral preparations, the ingredients described as additive in connection with oral preparations may be added.

The dosage and the frequency of administration may vary according to the desired therapeutic effect, the method of administration, the period of treatment, the age and body weight of the patient, and the like. However, the drugs of the present invention are usually administered to adults in a daily dose of 10 $\mu\text{g/kg}$ to 20 mg/kg .

One of the activities involved in an arteriosclerotic lesion (i.e. the activities regulating the development of arteriosclerosis) is the promotion or suppression of the apoptosis of vascular endothelial cells. Since it is known that, in vascular endothelial cells, the application of a shear stress tends to suppress the apoptosis of endothelial cells, the shear stress-responsive DNA of the present invention is considered to contain a gene and protein which exhibits a shear stress-dependent increase of expression in vascular endothelial cells and has an apoptosis-suppressing activity. Accordingly, by using this DNA containing a gene having an apoptosis-suppressing activity, a protein encoded by the DNA, a recombinant virus vector constructed by inserting the DNA into a vector, an antibody against the protein encoded by the DNA, and the like, the following applications can be made: (1) identification of the apoptosis sensitivity of cells, (2) regulation of the apoptosis of cells, and (3) screening of an agent for regulating the apoptosis of cells. These applications (1), (2) and (3) are described below in greater detail.

(1) Identification of the apoptosis sensitivity of cells

Now, the method for identifying the apoptosis sensitivity of cells using the shear stress-responsive DNA of the present invention or a protein encoded by the DNA is described below.

Apoptosis sensitivity means the degree of ease with which cells undergo apoptosis in response to an exogenous apoptotic stimulus, i.e. the degree of susceptibility of cells to the influence of an apoptotic stimulus. It is believed that this apoptosis sensitivity is defined according to whether the apoptotic signal in the cells is accompanied by a suppressive or promotive signal. The molecular entity thereof comprises a group of proteins involved in the suppression or promotion of apoptosis (e.g., apoptosis signal transduction molecule), i.e., the so-called apoptosis-related proteins. These apoptosis-related proteins include, for example, a

protein encoded by the DNA (A4RS-041) having the nucleotide sequence represented by SEQ ID NO:7 of the present invention, and a protein having the amino acid sequence represented by SEQ ID NO:8.

Hemodynamic physical forces applied to vascular endothelial cells include a shear stress resulting from a flow of blood with fixed directionality (i.e., a laminar flow) and applied in parallel with the direction of the blood flow, and a normal stress caused by a blood pressure and applied perpendicularly to the endothelium. Vascular endothelial cells are always subjected to both forces. Generally, the development of arteriosclerosis is suppressed in regions where the shear stress is greater than the normal stress. Conversely, the development of arteriosclerosis tends to occur in regions where the normal stress is greater than the shear stress. In fact, it has been reported that the apoptosis of vascular endothelial cells is suppressed by a shear stress resulting from a laminar flow. In the culture system (i.e., the micro carrier/spinner flask system) used to obtain the DNAs of the present invention, not only a shear stress due to a flow, but also a normal stress due to a centrifugal force caused by rotation is applied to endothelial cells. Some of the genes responding to a shear stress are modified by a normal stress, and others are not modified thereby. This difference in reactivity can be clarified by confirming the presence or absence of an increase of expression in HUVECs cultured in a parallel plate type culture apparatus or other apparatus which applies only a shear stress thereto. It is believed that at least the group of shear stress-responsive genes not modified by a normal stress act protectively against arteriosclerosis, and this gene group includes a gene and protein having an apoptosis-suppressing activity.

The endogenous transcription level of the DNA of the present invention having an apoptosis-suppressing activity, or the expression level of the protein of the present invention having an apoptosis-suppressing activity, or a structural change of the expressed protein may be detected using the DNA of the present invention having an apoptosis-suppressing activity, a DNA having the same sequence as 5 to 60 consecutive bases in the nucleotide sequence of the DNA, an antibody capable of recognizing the protein of the present invention having an

apoptosis-suppressing activity, or the like. Thus, the apoptosis sensitivity of cells can be identified.

Examples of the DNA used in the method for identifying apoptosis sensitivity, and an antibody capable of recognizing a protein encoded by the DNA include a DNA having the nucleotide sequence represented by SEQ ID NO:7, a DNA having the same sequence as 5 to 60 consecutive bases in the nucleotide sequence represented by SEQ ID NO:7, and an antibody capable of recognizing a protein having the amino acid sequence represented by SEQ ID NO:8.

The DNA of the present invention, a DNA having the same sequence as 5 to 60 consecutive bases in the nucleotide sequence of the DNA, and an antibody capable of recognizing the protein of the present invention having an apoptosis-suppressing activity, which are used in the above-described method, are effective as agents for identifying the apoptosis sensitivity of cells.

Since the apoptosis of vascular endothelial cells is promoted in an arteriosclerotic lesion, these agents can also be utilized as diagnostic agents for vascular diseases caused by arteriosclerosis with a view, for example, to identifying the arteriosclerotic lesion or predicting the risk of developing arteriosclerosis in the future.

The agent for identifying the apoptosis sensitivity of cells include, for example, an agent containing a DNA having the nucleotide sequence represented by SEQ ID NO:7, a DNA having the same sequence as 5 to 60 consecutive bases in the nucleotide sequence represented by SEQ ID NO:7, an antibody capable of recognizing a protein having the amino acid sequence represented by SEQ ID NO:8, or the like.

Since the DNAs of the present invention were obtained from human umbilical vein endothelial cells (HUVECs) as shear stress-responsive DNAs, it is desirable that the objective cells used for the identification of apoptosis sensitivity are vascular endothelial cells such as human primary cultured vascular endothelial cells and human umbilical vein endothelial cells (HUVECs). However, since apoptosis is a phenomenon universally observed in all types of

cells of the living body including vascular endothelial cells, the objective cells are not limited to vascular endothelial cells.

(2) Regulation of the apoptosis of cells

Since the DNA of the present invention is a shear stress-responsive gene which is known to exhibit an increase of expression in response to a shear stress and lead to the suppression of apoptosis, the DNA of the present invention or a DNA having the same sequence as 5 to 60 consecutive bases in the DNA may be involved in the suppression of apoptosis. On the other hand, when an antisense DNA having a nucleotide sequence complementary to the nucleotide sequence of each of these DNAs is used, the apoptosis of cells is promoted because the endogenous transcription or translation of the DNA is suppressed.

Similarly to the DNA of the present invention, the apoptosis of cells may also be regulated using a protein encoded by the DNA of the present invention or an antibody capable of recognizing the protein. Specifically, a protein having an apoptosis-suppressing activity is selected from various proteins encoded by the DNAs of the present invention, and the DNA encoding this protein is integrated into a virus vector to create a recombinant virus vector. Then, the apoptosis of cells or a tissue may be suppressed by introducing the recombinant virus vector into the cells or tissue and expressing the protein having an apoptosis-suppressing activity.

Moreover, the apoptosis of cells may be regulated by using an antibody capable of recognizing the aforesaid protein and thereby giving a positive or negative apoptosis-regulating signal to the cells.

Examples of the method for suppressing or promoting apoptosis include a method for promoting the apoptosis of cells by suppressing the endogenous transcription or translation of the DNA using a DNA having the nucleotide sequence represented by SEQ ID NO:7, a DNA having the same sequence as 5 to 60 consecutive bases in the nucleotide sequence represented by SEQ ID NO:7, or an antisense DNA having a nucleotide sequence complementary to the nucleotide sequence of each of these DNAs, for example, according to the antisense

technique; and a method for suppressing the apoptosis of cells by introducing the DNA into the cells and thereby accelerating the transcription of the DNA.

Moreover, they also include a method for suppressing the apoptosis of cells by increasing the intracellular expression level of a protein having the amino acid sequence represented by SEQ ID NO:8, using a recombinant virus vector containing a DNA having the nucleotide sequence represented by SEQ ID NO:7, a recombinant virus vector containing an RNA comprising a sequence homologous with the sense strand of the DNA having the nucleotide sequence represented by SEQ ID NO:7, or a recombinant virus vector capable of producing a protein having the amino acid sequence represented by SEQ ID NO:8.

Furthermore, since the amino acid sequence represented by SEQ ID NO:8 is considered to be a membrane protein on the basis of its structure, they also include a method for regulating the apoptosis of cells by subjecting the cells to the action of an antibody capable of recognizing a protein having the amino acid sequence represented by SEQ ID NO:8, stimulating the protein expressed on the cell surface, and thereby transducing a positive or negative apoptosis-regulating signal in the cells.

The DNA of the present invention, a DNA having the same sequence as 5 to 60 consecutive bases in the nucleotide sequence of the DNA, a recombinant virus vector capable of expressing the protein of the present invention having an apoptosis-suppressing activity, and an antibody capable of recognizing the protein of the present invention, which are used in the above-described methods, are effective as agents for regulating the apoptosis of cells. These agents can also be utilized as therapeutic agents for vascular diseases caused by arteriosclerosis.

The agents for regulating apoptosis include, for example, an agent containing a DNA having the nucleotide sequence represented by SEQ ID NO:7, a DNA having the same sequence as 5 to 60 consecutive bases in the nucleotide sequence represented by SEQ ID NO:7, an antisense DNA having a nucleotide sequence complementary to the nucleotide sequence of each of these DNAs, a recombinant virus vector containing a DNA having the nucleotide sequence represented by SEQ ID NO:7, a recombinant virus vector containing an

RNA comprising a sequence homologous with the sense strand of the DNA having the nucleotide sequence represented by SEQ ID NO:7, a recombinant virus vector capable of producing a protein having the amino acid sequence represented by SEQ ID NO:8, or an antibody capable of recognizing a protein having the amino acid sequence represented by SEQ ID NO:8.

Since the DNAs of the present invention were obtained from human umbilical vein endothelial cells (HUVECs) as shear stress-responsive DNAs, it is desirable that the objective cells used for the regulation of apoptosis are vascular endothelial cells such as human primary cultured vascular endothelial cells and human umbilical vein endothelial cells (HUVECs). However, since apoptosis is a phenomenon universally observed in all types of cells of the living body including vascular endothelial cells, the objective cells are not limited to vascular endothelial cells.

(3) Screening of an agent for regulating the apoptosis of cells

The methods for screening an agent for regulating the apoptosis of cells using the shear stress-responsive DNA of the present invention or a protein encoded by the DNA are described below.

One of the aforesaid screening methods is such that, when apoptosis is induced in an animal cell line exhibiting the Fas-dependent induction of apoptosis, a compound or protein which can suppress or promote apoptosis by regulating the endogenous transcription or translation of the DNA of the present invention is selected.

In particular, a compound or protein which can suppress apoptosis by promoting the endogenous transcription or translation of the DNA of the present invention is effective for the treatment of vascular diseases caused by arteriosclerosis. On the other hand, a compound or protein which can promote apoptosis by suppressing the endogenous transcription or translation of the DNA of the present invention is effective for the treatment of diseases based on abnormal proliferation of cells, such as cancer.

According to one exemplary method for screening an agent for regulating the apoptosis of cells using the DNA of the present invention, after a test material is made to act

on cells, an increase or decrease of the endogenous transcription level of a DNA having the nucleotide sequence represented by SEQ ID NO:7 is assayed using the DNA having the nucleotide sequence represented by SEQ ID NO:7, or a DNA having the same sequence as 5 to 60 consecutive bases in the nucleotide sequence represented by SEQ ID NO:7. Thus, an agent for suppressing or promoting the apoptosis of the cells can be screened.

Another of the aforesaid screening methods is such that, when an animal cell which has been transformed by introducing the DNA of the present invention so as to produce the protein of the present invention or a partial polypeptide of the protein is used, a compound or protein which can suppress the apoptosis of the cell by binding specifically to the cell is selected. In this method, the specific binding of a compound or protein can be detected by using an untransformed cell as a control. The agent obtained by this screening is also effective for the treatment of vascular diseases caused by arteriosclerosis.

According to one exemplary screening method using the protein of the present invention, a DNA having the nucleotide sequence represented by SEQ ID NO:7 is introduced into cells using a recombinant virus vector containing the DNA having the nucleotide sequence represented by SEQ ID NO:7, or a recombinant virus vector containing an RNA comprising a sequence homologous with the sense strand of the DNA having the nucleotide sequence represented by SEQ ID NO:7, to express a protein having the amino acid sequence represented by SEQ ID NO:8. By exposing the cells to a test material so as to contact the test material with the protein, an agent which binds specifically to the protein to change the activity of the protein is selected. Thus, an agent for suppressing or promoting the apoptosis of cells can be screened.

Alternatively, a DNA having the nucleotide sequence represented by SEQ ID NO:7 or a DNA encoding a protein having the amino acid sequence represented by SEQ ID NO:8 is inserted into a vector to construct a recombinant DNA. This recombinant DNA is introduced into a host cell, and the resulting transformant is cultured in a culture medium. By using the resulting culture to contact the protein in the culture with a test material, an agent which binds

specifically to the protein to change the activity of the protein is selected. Thus, an agent for suppressing or promoting the apoptosis of cells can be screened.

Alternatively, an isolated and purified protein having the amino acid sequence represented by SEQ ID NO:8 or a partial peptide of the protein having the amino acid sequence represented by SEQ ID NO:8 is used in an in vitro system. By contacting a test material with the protein or the peptide, an agent which binds specifically to the protein or peptide to cause a change in the activity of the protein is selected. Thus, an agent for suppressing or promoting the apoptosis of cells can be screened.

When an agent for suppressing or promoting apoptosis is screened by using an increase or decrease of the transcription level of a DNA having the nucleotide sequence represented by SEQ ID NO:7 in the cells as an index, the transcription level of the DNA may be analyzed according to a technique such as Northern hybridization, in situ hybridization, RNase protection assay or RT-PCR, by using a probe or primer comprising the DNA having the nucleotide sequence represented by SEQ ID NO:7, or a DNA having the same sequence as 5 to 60 consecutive bases in the nucleotide sequence represented by SEQ ID NO:7.

When an agent for suppressing or promoting apoptosis is screened by using the expression level of a protein having the amino acid sequence represented by SEQ ID NO:8 in the cells as an index, the expression level of the protein may be analyzed according to an immunological detection technique using an antibody capable of recognizing the protein having the amino acid sequence represented by SEQ ID NO:8.

The agents obtained by the above-described screening methods can be utilized as an agent for suppressing or promoting the apoptosis of cells.

Since the DNAs of the present invention were obtained from human umbilical vein endothelial cells (HUVECs) as shear stress-responsive DNAs, it is desirable that the objective cells used for the regulation of apoptosis are vascular endothelial cells such as human primary cultured vascular endothelial cells and human umbilical vein endothelial cells (HUVECs). However, since apoptosis is a phenomenon universally observed in all types of cells of the

living body including vascular endothelial cells, the objective cells are not limited to vascular endothelial cells.

As the vector used to express the DNA of the present invention in an animal cell and the method for introducing a recombinant vector, there may be employed any of the previously described methods.

As the immunological detection technique for assaying an increase or decrease of the expression level of the protein of the present invention using an antibody, there may be employed any of the previously described techniques.

As the host cell required in a screening system for detecting the suppression or promotion of apoptosis, there may be used any animal cell that exhibits the Fas-dependent induction of apoptosis. Examples thereof include suspension type cells such as Jurkat [J. Exp. Med., 152, 1709-19(1980)], HPB-ALL [Int. J. Cancer, 21, 166-170(1978)] and SKW6.4[Immunol. Lett., 7, 17-23(1983)]; and adhesion type cells such as HeLa and A673 [Arch. Biochem. Biophys., 230, 93-102(1984)].

One example of a substance for inducing Fas-dependent cell death in the aforesaid cell lines is the anti-human Fas monoclonal antibody CH-11 [J. Exp. Med., 169, 1747-1756(1989)]. Exemplary methods for inducing cell death are as follows. In the case of a suspension type cell, a cell suspension is diluted with a culture medium so as to have a density of about 10^6 cells/ml and added to a 24-well plate or a 96-well microtiter plate for the culture of animal cells. After the anti-human Fas monoclonal antibody is added to a concentration of 1 to 500 ng/ml, the plate is incubated in a CO₂ incubator at 37°C for several hours to 2 day and culture is carried out. In the case of an adhesion type cell, cells are inoculated onto a plate in advance. When cell death is to be induced, the culture medium is replaced by a culture medium containing the anti-human Fas monoclonal antibody, and the culture is continued in a CO₂ incubator at 37°C.

As the method for detecting the suppression or promotion of apoptosis, there may be employed, for example, a detection method in which the cells are stained with trypan blue, Giemsa stain or the like and observed under an optical microscope. In the case of adhesion

type cells, apoptosis causes cells to detach from the plate and float. Accordingly, the occurrence of apoptosis can be easily detected without staining. Also known is a detection method in which the cells are stained with a fluorochrome such as Hoechst 33342, Hoechst 33258 or propidium iodide and observed under a fluorescence microscope [Biomanual UP Series, New Experimental Methods for the Research of Apoptosis (in Japanese), Second Edition]. Moreover, there may also be employed biochemical methods such as a method involving the measurement of the activity of caspase activated in the process of apoptosis [J. Exp. Med., 183, 1957-1964(1996)], and MTT assay involving the measurement of mitochondrial dehydrogenase activity in living cells [J. Immunol. Methods, 16, 55-63(1983)]. Furthermore, a method for detecting a structural change of cell membrane using Annexin V [J. Exp. Med., 182, 1545-1556(1995)], and detection methods based on DNA fragmentation such as TUNEL method and Burton's method [Biomanual UP Series, New Experimental Methods for the Research of Apoptosis (in Japanese), Second Edition] are also known.

Examples

The present invention is more specifically described hereinbelow with reference to the following examples. However, the present invention is in no way to be limited to these examples.

Example 1

Construction of a cDNA library from HUVECs having a shear stress applied thereto

(1) Culture of HUVECs

Using F-12K medium (manufactured by Dainippon Pharmaceutical Co., Ltd.) containing 10% fetal calf serum, 1% penicillin (5,000 units/ml)/streptomycin (5 mg/ml) solution (manufactured by Life Technologies), 0.003% Endothelial Cell Growth Supplement (manufactured by Becton Dickinson), 0.01% heparin (manufactured by Wako Pure Chemical Industries Ltd.) and 0.14% NaHCO₃ (manufactured by Life Technologies), HUVECs were cultured and subcultured under the condition of 5% CO₂ and 37°C. The HUVECs used were purchased from Clonetics.

(2) Application of a shear stress to HUVECs

A suspension of 0.2 g of micro-carriers (Cytodex 3; manufactured by Amersham Pharmacia Biotech) in 10 ml of PBS buffer was transferred to a sterilized 50 ml tube, and centrifuged at 1,000 rpm for 3 minutes at room temperature. After the supernatant was removed, F12K medium was added. After the resulting suspension was centrifuged again and the supernatant was removed, the medium was added to make up to about 10 ml.

After the HUVECs obtained in the above culture step (1) were dissociated with trypsin/EDTA, about 2×10^6 HUVECs were suspended in 10 ml of the medium and mixed with the above-described micro-carriers. This mixture was transferred to a 200 ml spinner flask, and 15 ml of the medium was added to make a total volume of about 35 ml. The mixture was stirred at 50-60 rpm for 30 seconds and then allowed to stand for one hour. By repeating this stirring/standing procedure four times, HUVECs were made to adhere to the micro-carriers. Thereafter, a shear stress was applied to the cells by stirring the mixture at 160 rpm for a selected period of time.

(3) Preparation of RNA

Samples of 1.6×10^7 HUVECs having a shear stress applied thereto for 0.5 hour, 1 hour, 1.5 hours, 2 hours, 3 hours, 4 hours, 6 hours, 10 hours, and 20 hours respectively were prepared in the manner described in the above step (2). From each of the aforesaid nine samples of cells having different shear stress application times, total RNAs were prepared by the guanidine thiocyanate-cesium trifluoroacetate method [Methods in Enzymology, 154, 3(1987)]. 100 μ g each of these total RNAs from the nine samples were mixed to obtain 900 μ g of total RNA. This 900 μ g of total RNA was passed through an oligo-dT cellulose column (manufactured by Collaborative Research) to obtain 30.9 μ g of mRNA as poly(A)⁺ RNA.

(4) Construction of a cDNA library

Using 3.0 μ g of mRNA obtained in the above step (3), the synthesis of cDNA, the addition of BamHI adapter, and cleavage reaction with NotI were carried out according to the linker primer method [Hiroshi Nojima ed., "Methods for the Construction of Gene Libraries" (in Japanese)]. The resulting double-stranded cDNAs were ligated between BglII/NotI of the plasmid vector pAP3neo [Genes to Cells, 3, 459(1998)] so that 5'-terminus of the cDNAs was

always located on the BglIII site of the vector. Using the resulting ligation reaction solution, the plasmid was introduced into Escherichia coli MC1061A (Molecular Cloning, Second Edition) by electroporation. Thus, a cDNA library was constructed.

Example 2

Construction of a subtraction library

(1) Preparation of single-strand DNA

2 µg of the plasmid of the cDNA library obtained in Example 1 by amplification in MC1061A was introduced into Escherichia coli XL1-Blue MRF' (manufactured by Stratagene) by electroporation. After this Escherichia coli was suspended in 4.5 ml of SOC medium (Molecular Cloning, Second Edition) and incubated at 37°C for 1 hour with vigorous shaking, all of the resulting culture was added to 5.5 ml of LB medium (Molecular Cloning, Second Edition) containing 50 µg/ml ampicillin. After being incubated at 37°C for 5 hours with vigorous shaking, 5 ml of the resulting culture was inoculated into 45 ml of 2·YT medium (Molecular Cloning, Second Edition) containing ampicillin, and 1×10^{11} pfu of helper phage R408 [Gene, 45, 333(1986)] was added thereto. After being incubated at 37°C for 12 hours with vigorous shaking, the resulting culture was transferred to a sterilized tube and centrifuged at 10,000 rpm for 10 minutes at 4°C to precipitate the Escherichia coli. The phage-containing supernatant was transferred to a new sterilized tube and centrifuged again. The supernatant was passed through a sterilizing filter (manufactured by Millipore) having a pore diameter of 0.22 µm to remove the Escherichia coli completely. 2.5 ml of 10·DNase buffer [100 mM Tris-HCl (pH 7.5), 100 mM MgCl₂], 1 µl of 20 units/µl DNase I (manufactured by Nippon Gene Co., Ltd.) were added to 25 ml of the phage solution, and this mixture was reacted at 37°C for 30 minutes. Then, 1/4 volume of 20% polyethylene glycol (molecular weight 6,000)/2.5 M NaCl was added thereto and mixed well, following by standing at room temperature for 20 minutes. After this mixture was centrifuged at 10,000 rpm for 10 minutes at 4°C, the supernatant was removed completely. The resulting precipitate of phage was dissolved in 400 µl of TE [10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)], and 25 µl of 25 mg/ml Proteinase K and 4 µl of 10% SDS were added thereto,

following by reaction at 42°C for 1 hour. The reaction mixture was subjected to a phenol treatment, a phenol-chloroform treatment and a chloroform treatment, and then precipitated with ethanol. The resulting precipitate of single-strand phage DNA was dissolved in 30 µl of TE.

(2) Biotinylation of RNA

In the same manner as in Example 1, poly(A)⁺ RNA was prepared from HUVECs having no shear stress applied thereto (i.e., HUVECs made only to adhere to micro carriers). To 30 µg of this RNA was added distilled water so as to make a volume of 20 µl. Then, 30 µl of 1 µg/µl PHOTOPROBE biotin (manufactured by Vector Laboratories) was added thereto in the dark. After the tube was uncapped and placed on ice, the mixture was irradiated with light from a mercury vapor lamp disposed about 10 cm above the tube for 20 minutes to biotinylate the RNA, followed by the addition of 50 µl of 100 mM Tris·HCl (pH 9.5)/1 mM EDTA (pH 8.0). Then, 100 µl of water-saturated butanol was added thereto, followed by vigorous stirring. After this mixture was centrifuged at 14,000 rpm for 5 minutes at 4°C, the upper butanol layer was removed. This procedure was repeated two more times. 100 µl of chloroform was added to the aqueous layer, followed by vigorous stirring. After this mixture was centrifuged at 14,000 rpm for 5 minutes at 4°C, the aqueous layer was transferred to a new tube. After this procedure was repeated again, RNA was precipitated with ethanol. The recovered precipitate of RNA was dissolved in 20 µl of distilled water, and subjected again to the procedure for biotinylation. The biotinylated RNA was preserved at -80°C in the ethanol-precipitated state till use for hybridization.

(3) Hybridization of single-strand DNA with RNA

20 µg of the biotinylated RNA prepared in step (2) was recovered by centrifugation at 14,000 rpm for 15 minutes at 4°C, and dissolved in 8 µl of distilled water. To this solution, 12.5 µl of 2 x reaction buffer [80% formamide, 100 mM HEPES (pH 7.5), 2 mM EDTA (pH 8.0), 0.2% SDS], 2.5 µl of 2.5 M NaCl, 1 µl of 1 µg/µl poly(A) (manufactured by Amersham Pharmacia Biotech), and 1 µl (0.5 µg/µl) of the single-strand DNA prepared in step (1) from the cDNA library derived from HUVECs having a shear stress applied thereto were added so

as to make a total volume of 25 μ l. After this mixture was heated at 65°C for 10 minutes, it was quickly transferred to a heat block warmed at 42°C and incubated at 42°C for two nights to effect hybridization.

(4) Subtraction and rehybridization

After completion of the hybridization, 400 μ l of a buffer [500 mM NaCl, 50 mM HEPES (pH 7.5), 2 mM EDTA (pH 8.0)] was added to the reaction mixture. Then, 5 μ l of 2 μ g/ μ l streptavidin (manufactured by Life Technologies) was added thereto and mixed therewith. After this mixture was allowed to stand at room temperature for 5 minutes, it was subjected to a phenol-chloroform treatment. The aqueous layer was transferred to a new tube, and 5 μ l of fresh streptavidin was added thereto. After this mixture was allowed to stand at room temperature for 5 minutes, subtraction was carried out by subjecting it twice to a phenol-chloroform treatment and once to a chloroform treatment. The aqueous layer was placed in the upper chamber of a Millipore Filter UFCP3TK50 (manufactured by Millipore) and centrifuged at 10,000 rpm at 4°C until all of the solution passed into the lower chamber. After the solution was removed from the lower chamber, the filter was washed by adding 300 μ l of TE to the upper chamber and centrifuging the filter. After this procedure was repeated, single-strand DNA captured on the filter was recovered with 30 μ l of 1/10 TE. This single-strand DNA was dried under vacuum and dissolved in distilled water to make up to 9 μ l. After 10 μ g of the biotinylated RNA prepared in step (2) was precipitated with ethanol and recovered by centrifugation, 9 μ l of the above single-strand DNA solution was added to the precipitate. After the addition of 12.5 μ l of 2 x reaction buffer, 2.5 μ l of 2.5 M NaCl, and 1 μ l of poly(A), a second hybridization step was carried out in the same manner as in step (3), and subtraction was carried out in the above-described manner. Thereafter, single-strand DNA was recovered in a similar manner and subjected to a third subtraction step by hybridization with 10 μ g of the biotinylated RNA and a fourth subtraction step by using 5 μ g of the biotinylated RNA.

(5) Synthesis of double-strand DNA and its introduction into Escherichia coli

After four subtraction steps were successively carried out as described above, the resulting single-strand DNA was recovered in 30 μ l of 1/10 TE. To a 15 μ l portion thereof, 14 μ l of distilled water and 1 μ l of a 2 μ g/ μ l primer extension primer having the nucleotide sequence represented by SEQ ID NO:159 were added, followed by heating at 65°C for 10 minutes. After this mixture was allowed to stand at room temperature for 5 minutes so as to anneal the primer to single-strand DNA, 5 μ l of 10 x reaction buffer (attached to BcaBEST Dideoxy Sequencing Kit; manufactured by Takara Shuzo Co., Ltd.), 10 μ l of a 1 mM dNTP mixture, 0.5 μ l of 3 μ g/ μ l single-strand DNA-binding protein (manufactured by USB), 2 μ l of 2 units/ μ l BcaBEST DNA polymerase (manufactured by Takara Shuzo Co., Ltd.), and 2.5 μ l of distilled water were added thereto. This mixture was reacted at 65°C for 1 hour to synthesize double-strand DNA. After the addition of 50 μ l of distilled water, the reaction mixture was subjected to a phenol-chloroform treatment and a chloroform treatment. The resulting solution was concentrated by means of a Millipore Filter UFCP3TK50, and the double-strand DNA was finally dissolved in 20 μ l of TE. Using 1/5 volume of this solution, the double-strand DNA was introduced into Escherichia coli MC1061A by electroporation.

(6) Reverse subtraction

Escherichia coli MC1061A having the double-strand DNA introduced therein, which was obtained in step (5), was cultured, and plasmid DNA was prepared from the Escherichia coli. In the same manner as in step (1), this plasmid DNA was introduced into Escherichia coli XL1-Blue MRF' to prepare single-strand DNA. Two μ g of mRNA derived from HUVECs having a shear stress applied thereto was biotinylated in the manner described in step (2), and mixed with 2 μ g of the aforesaid single-strand DNA. To this mixture, 12.5 μ l of 2 x reaction buffer, 2.5 μ l of 2.5 M NaCl, 1 μ l of 1 μ g/ μ l poly(A), and 1 μ l of distilled water were added so as to make a total volume of 25 μ l. In the same manner as in step (3), this mixture was incubated at 42°C for two nights to carry out hybridization. Four hundred μ l of a buffer [500 mM NaCl, 50 mM HEPES (pH 7.5), 2 mM EDTA (pH 8.0)] was added to the reaction mixture. Then, 7 μ l of 2 μ g/ μ l streptavidin was added thereto and mixed therewith. After this mixture was allowed to stand at room temperature for 5 minutes, phenol-chloroform

was added thereto with vigorous mixing. After this mixture was centrifuged at 14,000 rpm for 7 minutes at room temperature, the aqueous layer was removed. Then, 400 µl of fresh TE was added thereto with vigorous mixing. After this mixture was centrifuged at 14,000 rpm for 7 minutes at room temperature, the aqueous layer was removed. This procedure was repeated two more times, so that single-strand DNA which did not hybridize with the biotinylated RNA was removed. After 400 µl of TE was added without mixing, the tube was heated at 95°C for 5 minutes in the uncapped state. Thereafter, by placing the tube on ice for 5 minutes to denature the DNA, the single-strand DNA having hybridized with the biotinylated RNA and present in the phenol-chloroform layer was separated from the biotinylated RNA. After the reaction mixture was vigorously mixed and centrifuged at 14,000 rpm for 7 minutes at room temperature, the aqueous layer was transferred to a new tube. The aqueous layer was subjected again to a phenol-chloroform treatment and then to a chloroform treatment. The aqueous layer containing the single-strand DNA was concentrated by means of a Millipore Filter UFCP3TK50, and the single-strand DNA was finally recovered in 30 µl of 1/10 TE. Fifteen µl of this solution was dried under vacuum, and dissolved in distilled water to make up to 9 µl. Five µg of mRNA derived from HUVECs having no shear stress applied thereto was biotinylated and recovered by precipitation with ethanol. To the precipitate was added 9 µl of the aforesaid single-strand DNA solution. Then, 12.5 µl of 2 x reaction buffer, 2.5 µl of 2.5 M NaCl, and 1 µl of poly(A) were added thereto, and normal subtraction was carried out in the same manner as steps (3) and (4).

That is, a subtraction library in which genes exhibiting an increase of expression in response to the application of a shear stress in HUVECs were concentrated was prepared by carrying out four successive subtraction steps, one reverse subtraction step, and one normal subtraction step.

Example 3

Obtaining of clones exhibiting an alteration of expression by Northern hybridization

Northern hybridization was carried out in order to select clones which are included in the subtraction library obtained in Example 2 and exhibit a shear stress-dependent increase of expression.

(1) Transfer of RNA to a membrane

According to the same procedure as in Example 1, total RNAs were obtained from HUVECs having a shear stress applied thereto and HUVECs having no shear stress applied thereto, respectively. To 4 µg of each total RNA was added distilled water so as to make a volume of 1.8 µl. Then, 0.8 µl of 10 x MOPS buffer [80 mM sodium acetate, 197 mM MOPS, 10 mM EDTA (pH 8.0)], 1.4 µl of a 35% formaldehyde solution (manufactured by Nacalai Tesque), and 4 µl of deionized formamide were added thereto. After this mixture was heated at 65°C for 15 minutes and then cooled rapidly by placing it on ice for 5 minutes, the total amount thereof was electrophoresed through 1 x MOPS/2% formaldehyde/1% agarose gel. After completion of the electrophoresis, the gel was washed with distilled water for 20 minutes, and this washing step was repeated three times to remove any formaldehyde from the gel. After the gel was soaked in 20xSSC (3 M NaCl, 0.3 M sodium citrate) for 30 minutes, RNA in the gel was transferred to a nylon membrane Biodyne A (manufactured by Pall BioSupport) according to a capillary transfer method using 20xSSC. After completion of the transfer, the RNA was fixed to the membrane by allowing the membrane to stand at 80°C for 2 hours.

(2) Labeling of probes

In the subtraction library obtained in Example 2, clones having an inserted DNA fragment of not less than 0.4 kb size were treated by cleaving the plasmid with SmaI and NotI to excise the inserted DNA fragment. The fragments thus obtained were purified using a QIAquick Gel Extraction Kit (manufactured by QIAGEN), and the procedure therefor was carried out according to the manual attached to the kit. Using about 50 ng of the purified DNA fragments as templates, the DNA fragments were labeled using a Random Primer DNA Labeling Kit

Ver. 2 (manufactured by Takara Shuzo Co., Ltd.) and [α - 32 P]dCTP (110 TBq/mmol; Amersham Pharmacia Biotech), and used as probes. The procedure therefor was carried out according to the manual attached to the kit.

(3) Hybridization and autoradiography

The membrane prepared in step (1) was placed in a hybridization bag, and a freshly prepared hybridization solution [50% formamide, 5 x Denhardt's, 5 x SSC, 0.1% SDS, denatured salmon DNA (0.1 mg/ml)] was added thereto. The hybridization bag was incubated at 42°C for 2 hours or more to carry out prehybridization. The probes prepared in step (2) were denatured by heating them at 95°C for 5 minutes and cooling them rapidly. These probes were mixed with a hybridization solution and added to the prehybridized membrane. The hybridization bag was incubated at 42°C for 24 hours or more to carry out hybridization. The membrane was taken out of the hybridization bag, placed in 2 x SSC/0.1% SDS, and slowly shaken at room temperature for 10 minutes to remove the hybridization solution as much as possible. Then, the membrane was washed in 0.15 x SSC/0.1% SDS at 42°C for 30 minutes, and this washing step was repeated twice. After completion of the washing steps, autoradiography was carried out by exposing an X-ray film to the membrane. A total of 1,026 clones were named A4RS-1 to A4RS-1026, respectively, and each of them was subjected to Northern hybridization. Thus, there were obtained 107 clones exhibiting a shear stress-dependent increase of expression.

Example 4

Identification of clones exhibiting alteration of expression

(1) Determination of nucleotide sequences

With respect to the clones which were ascertained to exhibit an increase of expression in response to the application of a shear stress in Example 3, their nucleotide sequences were determined by means of a 377 DNA Sequencer (manufactured by Perkin Elmer). For the determination of the nucleotide sequences, a Dye Primer Cycle Sequencing Kit (manufactured by Perkin Elmer) was used. The procedure therefor was carried out according to the attached manual. The clones exhibiting alteration of expression were identified by comparing the

resulting nucleotide sequences with the database GenBank. As a result, the 107 clones were classified into 88 types of genes. In these 88 genes, 5 genes which have been reported to exhibit the induction of expression by a shear stress stimulus in vascular endothelial cells, i.e. the genes encoding endothelin 1, monocyte chemotactic protein 1, heparin-binding EGF-like growth factor, thrombomodulin, and transforming growth factor β , are included. Accordingly, 83 genes with which the induction of expression by a shear stress stimulus in vascular endothelial cells had not yet been reported could be identified. These genes included 55 known genes and 28 novel genes. With respect to genes whose sequences are not identical with any of the full-length cDNAs included in the known sequences, but are identical only with expressed sequence tags (ESTs) alone, and genes whose sequences are not identical with any of the known sequences (i.e., novel genes), all ESTs included in the corresponding UniGene are joined together to construct as long sequences as possible on a computer. With respect to eight of the novel genes, full-length cDNAs were cloned from a cDNA library prepared with a λ phage vector in Example 5 that will be given later.

(2) Known genes exhibiting a shear stress-dependent increase of expression

When the nucleotide sequence of A4RS-016 was determined, this was identical with the sequence of thioredoxin reductase [Accession: X91247] (SEQ ID NO:1). The amino acid sequence encoded by this gene is shown as SEQ ID NO:2. Thioredoxin reductase is an enzyme reducing thioredoxin using NADPH, and participates in various physiological reactions such as control of intracellular antioxidation, signal transduction, and NO production. Its Northern blots exhibiting a shear stress-dependent increase of expression are shown in lane 1 of FIG. 1 and lane 1 of FIG. 3.

When the nucleotide sequence of A4RS-026 was determined, this was identical with the sequence of lipopolysaccharide-induced protein gene [Accession: Q51544] (SEQ ID NO:3). The amino acid sequence encoded by this gene is shown as SEQ ID NO:4. The protein encoded by this gene does not show substantial homology with other known proteins, and its function is unknown. Its Northern blot exhibiting a shear stress-dependent increase of expression is shown in lane 2 of FIG. 1.

When the nucleotide sequence of A4RS-040 was determined, this was identical with the sequence of spliceosome-associated protein (SAP145) [Accession: U41371] (SEQ ID NO:5). The amino acid sequence encoded by this gene is shown as SEQ ID NO:6. Its Northern blot exhibiting a shear stress-dependent increase of expression is shown in lane 3 of FIG. 1.

When the nucleotide sequence of A4RS-041 was determined, this was identical with the sequence of human proline-rich membrane protein (PRMP) [Accession: V50494] (SEQ ID NO:7). The amino acid sequence encoded by this gene is shown as SEQ ID NO:8. Only the sequence of PRMP is registered in a database, and its function is unknown. However, PRMP has substantial homology with rat neural membrane protein 35 (NMP35) [Molecular and Cellular Neuroscience, 11, 260(1998)] and the glutamate-binding subunit of NMDA receptor [Accession: W62612]. Although the function of NMP35 is not clearly known, it is expressed specifically in the brain, like the glutamate-binding subunit of NMDA receptor. From an analysis of hydrophilicity on the basis of its amino acid sequence, NMP35 is presumed to be a membrane protein. RPMP also has an extremely high degree of hydrophobicity and hence functions as a membrane protein. Its Northern blots exhibiting a shear stress-dependent increase of expression are shown in lane 4 of FIG. 1 and lane 2 of FIG. 3.

When the nucleotide sequence of A4RS-063 was determined, this was identical with the sequence of puromycin-sensitive aminopeptidase [Accession: AJ132583] (SEQ ID NO:9). The amino acid sequence encoded by this gene is shown as SEQ ID NO:10. Its Northern blots exhibiting a shear stress-dependent increase of expression are shown in lane 5 of FIG. 1 and lane 3 of FIG. 3.

When the nucleotide sequence of A4RS-096 was determined, this was identical with the sequence of human secreted protein gene 125 clone HSPAG15 [Accession: V59635] (SEQ ID NO:11). The amino acid sequence encoded by this gene is shown as SEQ ID NO:12. Only the sequence of this gene is registered in a bank, and its function is unknown. The protein encoded by this gene does not show substantial homology with other known proteins.

Its Northern blots exhibiting a shear stress-dependent increase of expression are shown in lane 6 of FIG. 1 and lane 4 of FIG. 3.

When the nucleotide sequence of A4RS-116 was determined, this was identical with the sequence of lamin C [Accession: M13451] (SEQ ID NO:13). The amino acid sequence encoded by this gene is shown as SEQ ID NO:14. Lamin C is a lining protein for the nuclear membrane and is one of the cytoskeleton forming factors. Its Northern blots exhibiting a shear stress-dependent increase of expression are shown in lane 7 of FIG. 1 and lane 5 of FIG. 3.

When the nucleotide sequence of A4RS-126 was determined, this was identical with the sequence of cytokine-response gene CR8 [Accession: T43383] (SEQ ID NO:15). The amino acid sequence encoded by this gene is shown as SEQ ID NO:16. Cytokine-response gene CR8, which is also called DEC1, is a transcription factor having a basic helix-loop-helix motif. In particular, it has high homology with a HES family of transcription factors participating in nerve differentiation. Its Northern blot exhibiting a shear stress-dependent increase of expression is shown in lane 8 of FIG. 1.

When the nucleotide sequence of A4RS-131 was determined, this was identical with the sequence of human enhancer of filamentation (HEF1) [Accession: L43821] (SEQ ID NO:17). The amino acid sequence encoded by this gene is shown as SEQ ID NO:18. HEF1 is a signal transduction molecule having an SH3 domain, having a FAK-binding activity, and participating in regulation of the cytoskeleton. Its Northern blot exhibiting a shear stress-dependent increase of expression is shown in lane 9 of FIG. 1.

When the nucleotide sequence of A4RS-148 was determined, this was identical with the sequence of interferon-induced 15-kDa protein gene [Accession: M21786] (SEQ ID NO:19). The amino acid sequence encoded by this gene is shown as SEQ ID NO:20. The protein encoded by this gene does not show substantial homology with other known proteins, and its function is unknown. Its Northern blot exhibiting a shear stress-dependent increase of expression is shown in lane 10 of FIG. 1.

When the nucleotide sequence of A4RS-154 was determined, this was identical with the sequence of LDL receptor [Accession: N60388] (SEQ ID NO:21). The amino acid sequence encoded by this gene is shown as SEQ ID NO:22. LDL receptor incorporates LDL, which is one of the causes for the formation of an arteriosclerotic lesion, under the endothelium. It has been reported that, when a shear stress is applied to cultured bovine aortic endothelial cells, the binding and incorporation of LDL via LDL receptor increases [Circulation, 76, 648(1987)]. Its Northern blot exhibiting a shear stress-dependent increase of expression is shown in lane 11 of FIG. 1.

When the nucleotide sequence of A4RS-174 was determined, this was identical with the sequence of peripheral myelin protein (PMP)-22 [Accession: Q32869] (SEQ ID NO:23). The amino acid sequence encoded by this gene is shown as SEQ ID NO:24. PMP-22 is a component of myelin present in the peripheral nervous system, and is a membrane protein having four transmembrane domains. Its Northern blot exhibiting a shear stress-dependent increase of expression is shown in lane 12 of FIG. 1.

When the nucleotide sequence of A4RS-175 was determined, this was identical with the sequence of tyrosine kinase receptor UFO/ArK [Accession: S65125] (SEQ ID NO:25). The amino acid sequence encoded by this gene is shown as SEQ ID NO:26. Its Northern blot exhibiting a shear stress-dependent increase of expression is shown in lane 13 of FIG. 1.

When the nucleotide sequence of A4RS-194 was determined, this was identical with the sequence of calcium-ATPase HK2 [Accession: M23115] (SEQ ID NO:27). The amino acid sequence encoded by this gene is shown as SEQ ID NO:28. Calcium-ATPase HK2 is present in the membranes of the endoplasmic reticulum within cells. Its Northern blot exhibiting a shear stress-dependent increase of expression is shown in lane 14 of FIG. 1.

When the nucleotide sequence of A4RS-197 was determined, this was identical with the sequence of human arginine-rich protein [Accession: M83751] (SEQ ID NO:29). The amino acid sequence encoded by this gene is shown as SEQ ID NO:30. The amino acid sequence encoded by this gene does not show substantial homology with other known proteins, and its function is unknown. However, it is suggested that this gene may be a kind

of proto-oncogene. Its Northern blot exhibiting a shear stress-dependent increase of expression is shown in lane 15 of FIG. 1.

When the nucleotide sequence of A4RS-260 was determined, this was identical with the sequence of KIAA0025 [Accession: D14695] (SEQ ID NO:31). The amino acid sequence encoded by this gene is shown as SEQ ID NO:32. The protein encoded by this gene does not show substantial homology with other known proteins, and its function is unknown. Its Northern blots exhibiting a shear stress-dependent increase of expression are shown in lane 16 of FIG. 1 and lane 6 of FIG. 3.

When the nucleotide sequence of A4RS-271 was determined, this was identical with the sequence of human high-mobility group phosphoprotein isoform I-C (HMGI-C) [Accession: U28749] (SEQ ID NO:33). The amino acid sequence encoded by this gene is shown as SEQ ID NO:34. Judging from its structure, HMGI-C is a transcription factor. Its Northern blots exhibiting a shear stress-dependent increase of expression are shown in lane 17 of FIG. 1 and lane 7 of FIG. 3.

When the nucleotide sequence of A4RS-307 was determined, this was identical with the sequence of PRAD1 [Accession: X59798] (SEQ ID NO:35). The amino acid sequence encoded by this gene is shown as SEQ ID NO:36. PRAD1 is a member of the cyclin family and is also called cyclin D1. Its Northern blots exhibiting a shear stress-dependent increase of expression are shown in lane 18 of FIG. 1 and lane 8 of FIG. 3.

When the nucleotide sequence of A4RS-355 was determined, this was identical with the sequence of KIAA0964 [Accession: AB023181] (SEQ ID NO:37). The amino acid sequence encoded by this gene is shown as SEQ ID NO:38. The protein encoded by this gene is judged to be the human ortholog of rat PSD-95/SAP90-associated protein-4 (SAPAP-4). SAPAP-4 is present in membranes and is considered to participate in the clustering of NMDA receptor. Its Northern blot exhibiting a shear stress-dependent increase of expression is shown in lane 19 of FIG. 1.

When the nucleotide sequence of A4RS-389 was determined, this was identical with the sequence of lamin A [Accession: M13452] (SEQ ID NO:39). The amino acid sequence

encoded by this gene is shown as SEQ ID NO:40. Lamin A is a lining protein for the nuclear membrane and is one of the cytoskeleton forming factors. Its Northern blots exhibiting a shear stress-dependent increase of expression are shown in panel 20 of FIG. 1 and panel 9 of FIG. 3.

When the nucleotide sequence of A4RS-391 was determined, this was identical with the sequence of non-muscle alpha actinin [Accession: U48734] (SEQ ID NO:41). The amino acid sequence encoded by this gene is shown as SEQ ID NO:42. Alpha actinin is one of the cytoskeleton forming factors. Its Northern blots exhibiting a shear stress-dependent increase of expression are shown in lane 21 of FIG. 1 and lane 10 of FIG. 3.

When the nucleotide sequence of A4RS-423 was determined, this was identical with the sequence of gamma-filamin [Accession: AF089841] (SEQ ID NO:43). The amino acid sequence encoded by this gene is shown as SEQ ID NO:44. Gamma-filamin is an actin filament crosslinking protein, and participates in filopodia formation by binding to low-molecular-weight GTP-binding proteins such as rac and rho. Its Northern blot exhibiting a shear stress-dependent increase of expression is shown in lane 22 of FIG. 1.

When the nucleotide sequence of A4RS-431 was determined, this was identical with the sequence of growth factor inducible immediate early gene product CYR61 [Accession: U62015] (SEQ ID NO:45). The amino acid sequence encoded by this gene is shown as SEQ ID NO:46. CYR61 is also called gigl, monocyte mature differentiation factor, or connective tissue growth factor-2, and is a secreted factor having a signal sequence at the amino-terminus. Its Northern blot exhibiting a shear stress-dependent increase of expression is shown in lane 23 of FIG. 1.

When the nucleotide sequence of A4RS-453 was determined, this was identical with the sequence of nuclear factor of activated T cells (NF-ATc) [Accession: U08015] (SEQ ID NO:47). The amino acid sequence encoded by this gene is shown as SEQ ID NO:48. NF-ATc is one of the components of a transcription factor. Its Northern blot exhibiting a shear stress-dependent increase of expression is shown in lane 24 of FIG. 1.

When the nucleotide sequence of A4RS-492 was determined, this was identical with the sequence of GLI Kruppel-related protein [Accession: M77698] (SEQ ID NO:49). The amino acid sequence encoded by this gene is shown as SEQ ID NO:50. GLI Kruppel-related protein, which is also called YY1, is a suppressively functioning transcription factor. Its Northern blot exhibiting a shear stress-dependent increase of expression is shown in lane 25 of FIG. 1.

When the nucleotide sequence of A4RS-507 was determined, this was identical with the sequence of human mRNA homologous to the p64 bovine chloride channel [Accession: Y12696] (SEQ ID NO:51). The amino acid sequence encoded by this gene is shown as SEQ ID NO:52. Only the sequence of this gene is reported, and its function is not clearly known. Its Northern blot exhibiting a shear stress-dependent increase of expression is shown in lane 26 of FIG. 1.

When the nucleotide sequence of A4RS-514 was determined, this was identical with the sequence of KIAA0080 [Accession: D38522] (SEQ ID NO:53). The amino acid sequence encoded by this gene is shown as SEQ ID NO:54. The protein encoded by this gene is judged to be the human ortholog of rat synaptotagmin XI that is a membrane protein. Its Northern blot exhibiting a shear stress-dependent increase of expression is shown in lane 27 of FIG. 1.

When the nucleotide sequence of A4RS-523 was determined, this was identical with the sequence of nicotinamide N-methyltransferase [Accession: U08021] (SEQ ID NO:55). The amino acid sequence encoded by this gene is shown as SEQ ID NO:56. Its Northern blot exhibiting a shear stress-dependent increase of expression is shown in lane 28 of FIG. 1.

When the nucleotide sequence of A4RS-544 was determined, this was identical with the sequence of H. sapiens mRNA for surface glycoprotein [Accession: Z50022] (SEQ ID NO:57). The amino acid sequence encoded by this gene is shown as SEQ ID NO:58. The protein encoded by this gene is a type I membrane protein. Its Northern blot exhibiting a shear stress-dependent increase of expression is shown in lane 29 of FIG. 1.

When the nucleotide sequence of A4RS-547 was determined, this was identical with the sequence of early growth response gene alpha(EGR-alpha) [Accession: S81439] (SEQ ID

NO:59). The amino acid sequence encoded by this gene is shown as SEQ ID NO:60. EGR-alpha is a transcription factor, and it has been reported that its homologue, EGR-1, is activated by a shear stress in endothelial cells [Arterioscler. Thromb. Vasc. Biol., 17, 2280(1997)]. Its Northern blot exhibiting a shear stress-dependent increase of expression is shown in lane 30 of FIG. 1.

When the nucleotide sequence of A4RS-557 was determined, this was identical with the sequence of SF2p33 [Accession: M69040] (SEQ ID NO:61). The amino acid sequence encoded by this gene is shown as SEQ ID NO:62. SF2p33 is a nuclear factor and is indispensable for the splicing of pre-mRNA. Its Northern blot exhibiting a shear stress-dependent increase of expression is shown in lane 31 of FIG. 1.

When the nucleotide sequence of A4RS-577 was determined, this was identical with the sequence of p66 shc [Accession: U73377] (SEQ ID NO:63). The amino acid sequence encoded by this gene is shown as SEQ ID NO:64. shc is a signal transduction molecule which transduces a stimulus from tyrosine kinase to ras. Its Northern blot exhibiting a shear stress-dependent increase of expression is shown in lane 32 of FIG. 1.

When the nucleotide sequence of A4RS-588 was determined, this was identical with the sequence of lysosomal acid lipase (LAL) [Accession: M74775] (SEQ ID NO:65). The amino acid sequence encoded by this gene is shown as SEQ ID NO:66. LAL, which is also called cholesteryl esterase, is an enzyme hydrolyzing cholesteryl esters incorporated into cells. If this gene is deficient, cholesteryl ester storage disease may be induced to cause arteriosclerosis. Its Northern blot exhibiting a shear stress-dependent increase of expression is shown in lane 33 of FIG. 1.

When the nucleotide sequence of A4RS-602 was determined, this was identical with the sequence of N^G,N^G-dimethylarginine dimethylaminohydrolase (DDAH) [Accession: AB001915] (SEQ ID NO:67). The amino acid sequence encoded by this gene is shown as SEQ ID NO:68. DDAH hydrolyzes N^G-monomethyl-L-arginine (MMA) and N^G,N^G-dimethyl-L-arginine (DMA) to citrullin. Since MMA and DMA are substrate analogs for NO synthase, they inhibit the synthesis of NO. That is, DDAH induces NO synthesis indirectly.

Its Northern blots exhibiting a shear stress-dependent increase of expression are shown in lane 34 of FIG. 1 and lane 11 of FIG. 3.

When the nucleotide sequence of A4RS-608 was determined, this was identical with the sequence of serum deprivation response (SDPR) [Accession: AF085481] (SEQ ID NO:69). The amino acid sequence encoded by this gene is shown as SEQ ID NO:70. For human SDPR, only its sequence is registered. It has been reported that the expression of its mouse ortholog, sdr, is induced by serum deprivation in NIH3T3. However, its function is unknown. Its Northern blot exhibiting a shear stress-dependent increase of expression is shown in lane 35 of FIG. 1.

When the nucleotide sequence of A4RS-612 was determined, this was identical with the sequence of regulator of G protein signaling (RGS3) [Accession: U27655] (SEQ ID NO:71). The amino acid sequence encoded by this gene is shown as SEQ ID NO:72. Its Northern blot exhibiting a shear stress-dependent increase of expression is shown in panel 36 of FIG. 1.

When the nucleotide sequence of A4RS-625 was determined, this was identical with the sequence of cytokine-inducible nuclear protein C-193 [Accession: X83703] (SEQ ID NO:73). The amino acid sequence encoded by this gene is shown as SEQ ID NO:74. In endothelial cells, this gene is expressed in response to inflammatory stimuli such as TNF- α and LPS. The amino acid sequence encoded by this gene does not show substantial homology with other known proteins, but has been proved to be a nuclear factor. Its Northern blot exhibiting a shear stress-dependent increase of expression is shown in lane 37 of FIG. 1.

When the nucleotide sequence of A4RS-666 was determined, this was identical with the sequence of laminin B1 chain [Accession: M61916] (SEQ ID NO:75). The amino acid sequence encoded by this gene is shown as SEQ ID NO:76. Laminin B1 chain is a glycoprotein and is a kind of extracellular matrix. It has been reported that, in bovine arterial endothelial cells, laminin protein is increased by the application of a shear stress [Laboratory Investigation, 73, 565(1995)]. Its Northern blot exhibiting a shear stress-dependent increase of expression is shown in lane 38 of FIG. 1.

When the nucleotide sequence of A4RS-668 was determined, this was identical with the sequence of Matrix Gla protein (MGP) [Accession: M58549] (SEQ ID NO:77). The amino acid sequence encoded by this gene is shown as SEQ ID NO:78. MGP is a kind of extracellular matrix. It has been reported that, in knockout mice of this gene, calcification occurs in arteries and cartilages and results in death [Nature, 386, 78(1997)]. Its Northern blot exhibiting a shear stress-dependent increase of expression is shown in lane 39 of FIG. 1.

When the nucleotide sequence of A4RS-674 was determined, this was identical with the sequence of PTX3 (SEQ ID NO:79). The amino acid sequence encoded by this gene is shown as SEQ ID NO:80. PTX3 is a member of the pentraxin family, and is a secreted factor having a signal sequence at the amino-terminus. It has been reported that, in vascular endothelial cells and monocytes, this gene is expressed in response to inflammatory stimuli such as IL-1 and TNF- α . Its Northern blot exhibiting a shear stress-dependent increase of expression is shown in lane 40 of FIG. 1.

When the nucleotide sequence of A4RS-682 was determined, this was identical with the sequence of connective tissue growth factor [Accession: X78947] (SEQ ID NO:81). The amino acid sequence encoded by this gene is shown as SEQ ID NO:82. Connective tissue growth factor is a secreted factor having a signal sequence at the amino-terminus, and its expression in developed arteriosclerotic lesions has been reported [Circulation, 95, 831(1997)]. Its Northern blot exhibiting a shear stress-dependent increase of expression is shown in lane 41 of FIG. 1.

When the nucleotide sequence of A4RS-751 was determined, this was identical with the sequence of FLI-1 [Accession: Q50644] (SEQ ID NO:83). The amino acid sequence encoded by this gene is shown as SEQ ID NO:84. FLI-1, which is also called ERGB, is a transcription factor belonging to the ETS family. Its Northern blot exhibiting a shear stress-dependent increase of expression is shown in lane 42 of FIG. 2.

When the nucleotide sequence of A4RS-781 was determined, this was identical with the sequence of HLA-E [Accession: X56841] (SEQ ID NO:85). The amino acid sequence encoded by this gene is shown as SEQ ID NO:86. HLA-E is a kind of MHC class I protein.

Its Northern blot exhibiting a shear stress-dependent increase of expression is shown in lane 43 of FIG. 2.

When the nucleotide sequence of A4RS-784 was determined, this was identical with the sequence of plasminogen activator inhibitor (PAI) [Accession: M16006] (SEQ ID NO:87). The amino acid sequence encoded by this gene is shown as SEQ ID NO:88. PAI acts antagonistically against plasminogen activator. It has been reported that its expression is decreased by the application of a shear stress [Blood, 87, 2314(1996)]. Its Northern blots exhibiting a shear stress-dependent increase of expression are shown in lane 44 of FIG. 2 and lane 12 of FIG. 3.

When the nucleotide sequence of A4RS-817 was determined, this was identical with the sequence of keratin 18 [Accession: M26326] (SEQ ID NO:89). The amino acid sequence encoded by this gene is shown as SEQ ID NO:90. Keratin 18 is a kind of intermediate filament. Its Northern blot exhibiting a shear stress-dependent increase of expression is shown in lane 45 of FIG. 2.

When the nucleotide sequence of A4RS-818 was determined, this was identical with the sequence of human secreted protein gene 5 clone HELDY41 [Accession: V34315] (SEQ ID NO:91). The amino acid sequence encoded by this gene is shown as SEQ ID NO:92. The amino acid sequence encoded by this gene coincides with a partial sequence of human hedgehog interacting protein [Accession: W56538]. Its Northern blot exhibiting a shear stress-dependent increase of expression is shown in lane 46 of FIG. 2.

When the nucleotide sequence of A4RS-914 was determined, this was identical with the sequence of monocyte-derived neutrophil-activating protein (MONAP) [Accession: M26383] (SEQ ID NO:93). The amino acid sequence encoded by this gene is shown as SEQ ID NO:94. MONAP is also called interleukin 8 (IL-8), and its relation with the development of arteriosclerosis is strongly suggested. In fact, its strong expression in an mRNA level and in a protein level has been reported in macrophages derived from arteriosclerotic plaques [Arterioscler. Thromb. Vasc. Biol., 16, 1007(1996)]. Its Northern blot exhibiting a shear stress-dependent increase of expression is shown in lane 47 of FIG. 2.

When the nucleotide sequence of A4RS-929 was determined, this was identical with the sequence of MUC18 glycoprotein [Accession: M28882] (SEQ ID NO:95). The amino acid sequence encoded by this gene is shown as SEQ ID NO:96. MUC18, which is also called Mel-CAM or CD146, is a cell adhesion factor having an immunoglobulin-like domain. Its Northern blot exhibiting a shear stress-dependent increase of expression is shown in lane 48 of FIG. 2.

When the nucleotide sequence of A4RS-935 was determined, this was identical with the sequence of nuclear speckle-type protein (SPOP) [Accession: AJ000644] (SEQ ID NO:97). The amino acid sequence encoded by this gene is shown as SEQ ID NO:98. SPOP is a nuclear factor which is considered to interact with splicing factors. Its Northern blot exhibiting a shear stress-dependent increase of expression is shown in lane 49 of FIG. 2.

When the nucleotide sequence of A4RS-938 was determined, this was identical with the sequence of thrombospondin (TSP) [Accession: X14787] (SEQ ID NO:99). The amino acid sequence encoded by this gene is shown as SEQ ID NO:100. TSP is a glycoprotein functioning as an extracellular matrix, and has an inhibitory effect on carcinogenesis and angiogenesis. Its Northern blot exhibiting a shear stress-dependent increase of expression is shown in lane 50 of FIG. 2.

When the nucleotide sequence of A4RS-939 was determined, this was identical with the sequence of caveolin [Accession: Z18951] (SEQ ID NO:101). The amino acid sequence encoded by this gene is shown as SEQ ID NO:102. Caveolin is a principal component of caveolae present in the cell membrane. It has been reported that caveolin participates in the control of NO production by interacting with nitric oxide (NO) synthase [J. Biol. Chem., 273, 34724(1998)]. Its Northern blot exhibiting a shear stress-dependent increase of expression is shown in lane 51 of FIG. 2.

When the nucleotide sequence of A4RS-945 was determined, this was identical with the sequence of human BENE mRNA [Accession: U17077] (SEQ ID NO:103). The amino acid sequence encoded by this gene is shown as SEQ ID NO:104. BENE is a membrane protein having homology with T cell surface glycoprotein MAL. Since its expression in

endothelial cells is increased by lysophosphatidyl choline (lysoPC) that is a component of oxidized lipoproteins, its relation with arteriosclerosis is suggested [J. Biochemistry, 123, 1119(1998)]. Its Northern blot exhibiting a shear stress-dependent increase of expression is shown in lane 52 of FIG. 2.

When the nucleotide sequence of A4RS-947 was determined, this was identical with the sequence of 1,4-alpha-glucan branching enzyme [Accession: L07956] (SEQ ID NO:105). The amino acid sequence encoded by this gene is shown as SEQ ID NO:106. Its Northern blot exhibiting a shear stress-dependent increase of expression is shown in lane 53 of FIG. 2.

When the nucleotide sequence of A4RS-948 was determined, this was identical with the sequence of ferritin H [Accession: M11146] (SEQ ID NO:107). The amino acid sequence encoded by this gene is shown as SEQ ID NO:108. Its Northern blot exhibiting a shear stress-dependent increase of expression is shown in lane 54 of FIG. 2.

When the nucleotide sequence of A4RS-949 was determined, this was identical with the sequence of human PAST (HPAST) [Accession: AF001434] (SEQ ID NO:109). The amino acid sequence encoded by this gene is shown as SEQ ID NO:110. HPAST has homology with PAST-1 that is a fly-derived glycoprotein. Its Northern blot exhibiting a shear stress-dependent increase of expression is shown in lane 55 of FIG. 2.

(3) Novel partial-length genes exhibiting a shear stress-dependent increase of expression

When the nucleotide sequence of A4RS-011 was determined, this was identical with a group of ESTs included in UniGene Hs. 71475. The sequence represented by SEQ ID NO:111 could be obtained by joining the corresponding ESTs together. The amino acid sequence encoded by this sequence is shown as SEQ ID NO:112. The amino acid sequence encoded by this sequence does not show substantial homology with other known proteins. Its Northern blot exhibiting a shear stress-dependent increase of expression is shown in lane 56 of FIG. 2.

When the nucleotide sequence of A4RS-115 was determined, this was identical with a group of ESTs included in UniGene Hs. 3742. The sequence represented by SEQ ID NO:113 could be obtained by joining the corresponding ESTs together. The amino acid sequence

encoded by this sequence is shown as SEQ ID NO:114. This gene has very high homology with rat SEC61 [Accession: M96630] and is considered to be the human ortholog thereof. Its Northern blots exhibiting a shear stress-dependent increase of expression are shown in lane 57 of FIG. 2 and lane 13 of FIG. 3.

When the nucleotide sequence of A4RS-143 was determined, this was identical with a group of ESTs included in UniGene Hs. 5307. The sequence represented by SEQ ID NO:115 could be obtained by joining the corresponding ESTs together. This sequence does not have an ORF consisting of 50 or more amino acids and is hence considered to be a non-translational region. Its Northern blots exhibiting a shear stress-dependent increase of expression are shown in lane 58 of FIG. 2 and lane 14 of FIG. 3.

When the nucleotide sequence of A4RS-171 was determined, no sequence identical exactly with it was found in the data banks. The nucleotide sequence is shown as SEQ ID NO:116. This sequence does not have an ORF consisting of 50 or more amino acids and is hence considered to be a non-translational region. Its Northern blot exhibiting a shear stress-dependent increase of expression is shown in lane 59 of FIG. 2.

When the nucleotide sequence of A4RS-193 was determined, this was identical with a group of ESTs included in UniGene Hs. 112157. The sequence represented by SEQ ID NO:117 could be obtained by joining the corresponding ESTs together. The amino acid sequence encoded by this sequence is shown as SEQ ID NO:118. The protein encoded by this sequence does not show substantial homology with other known proteins. Its Northern blots exhibiting a shear stress-dependent increase of expression are shown in lane 60 of FIG. 2 and lane 15 of FIG. 3.

When the nucleotide sequence of A4RS-280 was determined, this was identical with a group of ESTs included in UniGene Hs. 109017. The sequence represented by SEQ ID NO:119 could be obtained by joining the corresponding ESTs together. The amino acid sequence encoded by this sequence is shown as SEQ ID NO:120. This gene has as high as 87% homology with human ras-like protein TC10 [Accession: M31470] and is considered to be a novel human low-molecular-weight GTP-binding protein. Its Northern blots exhibiting a

shear stress-dependent increase of expression are shown in lane 61 of FIG. 2 and lane 16 of FIG. 3.

When the nucleotide sequence of A4RS-402 was determined, this was identical with a group of ESTs included in UniGene Hs. 181077. The sequence represented by SEQ ID NO:121 could be obtained by joining the corresponding ESTs together. The amino acid sequence encoded by this sequence is shown as SEQ ID NO:122. Its Northern blots exhibiting a shear stress-dependent increase of expression are shown in lane 62 of FIG. 2 and lane 17 of FIG. 3.

When the nucleotide sequence of A4RS-533 was determined, this was identical with EST clones R07925 and T86046. The sequence represented by SEQ ID NO:123 could be obtained by joining the corresponding ESTs together. The amino acid sequence encoded by this sequence is shown as SEQ ID NO:124. The amino acid sequence encoded by this sequence does not show substantial homology with other known proteins. Its Northern blot exhibiting a shear stress-dependent increase of expression is shown in lane 63 of FIG. 2.

When the nucleotide sequence of A4RS-604 was determined, this was identical with a group of ESTs included in UniGene Hs. 34160. The sequence represented by SEQ ID NO:125 could be obtained by joining the corresponding ESTs together. The amino acid sequence encoded by this sequence is shown as SEQ ID NO:126. The protein encoded by this sequence does not show substantial homology with other known proteins. Its Northern blots exhibiting a shear stress-dependent increase of expression are shown in lane 64 of FIG. 2 and lane 18 of FIG. 4.

When the nucleotide sequence of A4RS-615 was determined, this was identical with a group of ESTs included in UniGene Hs. 193974. The sequence represented by SEQ ID NO:127 could be obtained by joining the corresponding ESTs together. The amino acid sequence encoded by this sequence is shown as SEQ ID NO:128. The protein encoded by this sequence does not show significant homology with other known proteins. Its Northern blot exhibiting a shear stress-dependent increase of expression is shown in lane 65 of FIG. 2.

When the nucleotide sequence of A4RS-619 was determined, this was identical with a group of ESTs included in UniGene Hs. 14512. The sequence represented by SEQ ID NO:129 could be obtained by joining the corresponding ESTs together. This sequence does not have an ORF consisting of 50 or more amino acids and is hence considered to be a non-translational region. Its Northern blot exhibiting a shear stress-dependent increase of expression is shown in lane 66 of FIG. 2.

When the nucleotide sequence of A4RS-626 was determined, no sequence identical exactly with it was found in the data banks. The nucleotide sequence is shown as SEQ ID NO:130. This sequence does not have an ORF consisting of 50 or more amino acids and is hence considered to be a non-translational region. Its Northern blots exhibiting a shear stress-dependent increase of expression are shown in lane 67 of FIG. 2 and lane 19 of FIG. 4.

When the nucleotide sequence of A4RS-676 was determined, this was identical with a group of ESTs included in UniGene Hs. 8881. The sequence represented by SEQ ID NO:131 could be obtained by joining the corresponding ESTs together. This sequence does not have an ORF consisting of 50 or more amino acids and is hence considered to be a non-translational region. Its Northern blot exhibiting a shear stress-dependent increase of expression is shown in lane 68 of FIG. 2.

When the nucleotide sequence of A4RS-679 was determined, no sequence identical exactly with it was found in the data banks. The nucleotide sequence is shown as SEQ ID NO:132. This sequence does not have an ORF consisting of 50 or more amino acids and is hence considered to be a non-translational region. Its Northern blot exhibiting a shear stress-dependent increase of expression is shown in lane 69 of FIG. 2.

When the nucleotide sequence of A4RS-737 was determined, no sequence identical exactly with it was found in the data banks. The nucleotide sequence is shown as SEQ ID NO:133. This sequence does not have an ORF consisting of 50 or more amino acids and is hence considered to be a non-translational region. Its Northern blot exhibiting a shear stress-dependent increase of expression is shown in lane 70 of FIG. 2.

When the nucleotide sequence of A4RS-780 was determined, this was identical with a group of ESTs included in UniGene Hs. 34489. The sequence represented by SEQ ID NO:134 could be obtained by joining the corresponding ESTs together. This sequence does not have an ORF consisting of 50 or more amino acids and is hence considered to be a non-translational region. Its Northern blot exhibiting a shear stress-dependent increase of expression is shown in lane 71 of FIG. 2.

When the nucleotide sequence of A4RS-826 was determined, this was identical with a group of ESTs included in UniGene Hs. 7348. The sequence represented by SEQ ID NO:135 could be obtained by joining the corresponding ESTs together. The amino acid sequence encoded by this sequence is shown as SEQ ID NO:136. The protein encoded by this sequence does not show substantial homology with other known proteins. Its Northern blot exhibiting a shear stress-dependent increase of expression is shown in lane 72 of FIG. 2.

When the nucleotide sequence of A4RS-916 was determined, this was identical with a group of ESTs included in UniGene Hs. 105695. The sequence represented by SEQ ID NO:137 could be obtained by joining the corresponding ESTs together. The amino acid sequence encoded by this sequence is shown as SEQ ID NO:138. The protein encoded by this sequence does not show substantial homology with other known proteins. Its Northern blots exhibiting a shear stress-dependent increase of expression are shown in lane 73 of FIG. 2 and lane 20 of FIG. 4.

When the nucleotide sequence of A4RS-933 was determined, this was identical with EST clone AI391599. The sequence represented by SEQ ID NO:139 could be obtained by joining the corresponding ESTs together. The amino acid sequence encoded by this sequence is shown as SEQ ID NO:140. The protein encoded by this sequence does not show substantial homology with other known proteins. Its Northern blot exhibiting a shear stress-dependent increase of expression is shown in lane 74 of FIG. 2.

When the nucleotide sequence of A4RS-943 was determined, this was identical with a group of ESTs included in UniGene Hs. 186838. The sequence represented by SEQ ID NO:141 could be obtained by joining the corresponding ESTs together. The amino acid

sequence encoded by this sequence is shown as SEQ ID NO:142. The amino acid sequence encoded by this sequence has a zinc finger motif and shows 67% homology with bird-derived zinc finger 5 protein [Accession: U51640]. Its Northern blot exhibiting a shear stress-dependent increase of expression is shown in lane 75 of FIG. 2.

Example 5

Cloning of full-length cDNAs

With respect to the novel DNAs exhibiting a shear stress-dependent increase of expression which were obtained in Example 3, the length of the insert was significantly shorter than the size of mRNA detected by Northern blotting in most cases. That is, the clones obtained from the subtraction library were judged to be partial cDNA fragments and not full-length cDNAs. Accordingly, with respect to eight of the novel DNAs, their full-length cDNAs were obtained again from a cDNA library.

(1) Construction of a cDNA library using a λ phage vector

Three point two μg of oligo(dT)-XhoI primer (SEQ ID NO:160) was added to 4.8 μg of the HUVEC-derived poly(A)⁺ RNA obtained in Example 1. Then, distilled water was added thereto so as to make a volume of 6.8 μl . This solution was heated at 70°C for 10 minutes and then cooled rapidly by placing it on ice. To this solution, 4 μl of 5 x reverse transcriptase reaction buffer (attached to the enzyme), 2 μl of 100 mM DTT, 1.2 μl of a mixed dNTP solution [10 mM dATP, 10 mM dGTP, 10 mM dTTP, 5 mM 5-methyl dCTP], and 1 μl of [α -³²P]dATP (110 TBq/mmol; manufactured by Amersham Pharmacia Biotech) as a tracer were added on ice. After this mixture was kept at 37°C for 2 minutes, 5 μl of Superscript II RNase H⁻ Reverse Transcriptase (1,000 units; manufactured by Life Technologies) was added thereto and reacted at 44°C for 1 hour to synthesize cDNA. After the reaction was stopped by the addition of 0.8 μl of 0.5 M EDTA (pH 8.0), the reaction mixture was subjected to a phenol-chloroform treatment and a chloroform treatment, and precipitated with ethanol to recover a cDNA-mRNA hybrid. After the precipitate was dissolved in 17 μl of distilled water, 5 μl of 5 x reaction buffer (attached to the enzyme), 2.5 μl of 100 μM dGTP, and 0.5 μl of 15 units/ μl terminal deoxynucleotidyl transferase (manufactured by Life Technologies) were

added thereto. This mixture was reacted at 37°C for 30 minutes to add oligo-dG to the 3'-terminus of cDNA. After the reaction was stopped by the addition of 5 µl of 0.5 M EDTA (pH 8.0), the reaction mixture was subjected to a phenol-chloroform treatment and a chloroform treatment, and precipitated with ethanol. After the resulting precipitate was dissolved in 20.7 µl of distilled water, 1.5 µl of reaction buffer A [200 mM Tris·HCl (pH 8.75), 100 mM KCl, 100 mM (NH₄)₂SO₄, 20 mM MgSO₄, 1% Triton X-100, 1 mg/ml BSA], 1.5 µl of reaction buffer B [200 mM Tris·HCl (pH 9.2), 600 mM KCl, 20 mM MgCl₂], 0.3 µg of oligo(dC)-NotI primer (SEQ ID NO:161), 0.75 µl of a 10 mM mixed dNTP solution, and 1.5 µl of 10 mM β-NAD were added thereto so as to make a total volume of 27.45 µl. After this mixture was kept at 55°C for 5 minutes, 1.5 µl of 5 units/µl ExTaq DNA polymerase (manufactured by Takara Shuzo Co., Ltd.), 0.75 µl of 100 units/µl Ampligase (manufactured by Epicentre), and 0.3 µl of 5 units/µl Hybridase (manufactured by Epicentre) were added thereto. Using a Thermal Cycler DNA Engine (manufactured by MJ Research), the temperature of this mixture was slowly reduced from 55°C to 35°C at a rate of 0.3°C per minute. Thereafter, the mixture was kept at 35°C for 15 minutes to anneal the primer to the template single-stranded cDNA. Thereafter, the mixture was kept at 72°C for 15 minutes to carry out the extension reaction of second-strand DNA. By repeating this annealing/extension cycle three more times, mRNA was degraded to make double-stranded cDNA. To the reaction mixture, 0.5 µl of 0.5 M EDTA (pH 8.0), 0.5 µl of 10% SDS, and 0.5 µl of 20 µg/µl Proteinase K were added. Then, the reaction mixture was kept at 45°C for 15 minutes to stop the reaction and inactivate the enzyme. Thereafter, the reaction mixture was subjected to a phenol-chloroform treatment and a chloroform treatment, and precipitated with ethanol. The resulting precipitate was dissolved in 44 µl of distilled water. Then, 5 µl of 10 x reaction buffer (attached to the enzyme) and 1 µl of XhoI (10 units/µl; manufactured by Takara Shuzo Co., Ltd.) were added thereto and reacted at 37°C for 2 hours to cleave the XhoI site in the oligo(dT)-XhoI primer. Then, 0.5 µl of 5 M NaCl and 1 µl of NotI (10 units/µl; manufactured by Takara Shuzo Co., Ltd.) were added to the reaction mixture and reacted at 37°C for 2 hours to cleave the NotI site in the oligo(dC)-NotI primer. In order to remove short cDNA

fragments of not greater than 400 bp size, and unreacted primers and nucleotides, the reaction mixture was placed on a SizeSep-400 spun column (manufactured by Amersham Pharmacia Biotech) equilibrated with TE buffer, and centrifuged at 400 x g for 2 minutes. The resulting eluate was purified by subjecting it to a phenol-chloroform treatment and a chloroform treatment. Eight µl of 10 x reaction buffer (manufactured by Takara Shuzo Co., Ltd.), 62 µl of distilled water, and 50 units (5 µl) of XhoI were added to 5 µg (5 µl) of cloning vector λZAPII (manufactured by Stratagene), and reacted at 37°C for 4 hours. Then, 1 µl of 5 M NaCl and 50 units (5 µl) of NotI were added to the reaction mixture, and further reacted at 37°C for 4 hours. Thus, the XhoI and NotI sites of the vector were cleaved. Then, 9 µl of 10 x reaction buffer (attached to the enzyme) and 0.025 unit of temperature-sensitive alkaline phosphatase (manufactured by Life Technologies) were added to the reaction mixture and reacted at 65°C for 15 minutes to dephosphorylate the 5'-termini of the XhoI-cleaved end and NotI-cleaved end of the vector. After the reaction was stopped by the addition of 10 µl of a reaction stopper (attached to the enzyme), the reaction mixture was subjected to a phenol-chloroform treatment and a chloroform treatment, and the vector was recovered by ethanol precipitation. The aforesaid purified cDNA was added to 0.25 µg of the vector, followed by ethanol precipitation. The recovered vector DNA and cDNA were dissolved in 4 µl of a ligase buffer [100 mM Tris-HCl (pH 7.6), 5 mM MgCl₂, 300 mM NaCl], and 4 µl of solution B in a Ligation Kit Ver. 1 (manufactured by Takara Shuzo Co., Ltd.). This mixture was reacted at 26°C for 10 minutes to ligate cDNA to vector DNA. Using 4 µl portions of the reaction mixture, packaging was carried out using a λ Phage Packaging Extract Gigapack β Gold (manufactured by Stratagene). Specifically, reagents were used and the procedure for packaging was carried out according to the manual attached to the kit. Escherichia coli XL1-Blue MRF' strain was infected with the resulting phage, and its titer was measured. Moreover, the phage was multiplied on a plate and recovered in SM buffer (its composition is described in the manual of Stratagene). Thus, the cDNA library was amplified once to obtain a final cDNA library. Specifically, the procedures for titer measurement and library amplification were carried out according to the manual attached to the λ phage packaging kit.

(2) Obtaining of full-length cDNAs by plaque hybridization

With respect to the library constructed in step (1), plaque DNAs were blotted to a nylon membrane Hybond N+ (manufactured by Amersham Pharmacia Biotech). The plasmids derived from the subtraction library obtained in Example 2 were used as templates, and primers specific for each genes were synthesized and used. After a PCR DIG labeling mix (manufactured by Boehringer Mannheim) was added, PCR was carried out to amplify and label each gene-specific fragments. Using these DNA fragments as probes, hybridization and the detection of positive plaques were carried out according to the manual of Boehringer Mannheim. Positive plaques were amplified in SM buffer and formed into plasmids using helper phage ExAssist (manufactured by Stratagene). Specifically, the procedure for plasmid formation was carried out according to the manual of Stratagene.

(2) Determination of nucleotide sequence

The nucleotide sequence of each of the cDNA clones thus obtained was determined by means of a 377 DNA Sequencer (manufactured by Perkin Elmer). Specifically, the determination of the nucleotide sequence was carried out with a Dye Primer Cycle Sequencing FS Ready Reaction Kit according to the manual attached to the Kit (manufactured by Perkin Elmer). Moreover, this nucleotide sequence was translated into an amino acid sequence on a three-frame basis and examined for the presence of an open reading frame (ORF).

(3) Homology analysis of full-length cDNAs

①A4RS-002

With respect to the full-length cDNA clone pfA4RS-002-1 obtained as a result of plaque hybridization, the whole nucleotide sequence of cDNA was determined and the resulting nucleotide sequence is shown as SEQ ID NO:143. Escherichia coli DH5 α strain having clone pfA4RS-002-1 introduced therein (Escherichia coli DH5 α /pfA4RS-002-1) was internationally deposited on August 5, 1999 with the National Institute of Bioscience and Human-Technology, the Agency of Industrial Science and Technology (Higashi 1-1-3, Tsukuba City, Ibaraki Prefecture, Japan) under the Budapest Treaty, and assigned the

accession number FERM BP-6822. In the nucleotide sequence of A4RS-002, an ORF consisting of 390 amino acids was observed (its amino acid sequence is shown as SEQ ID NO:144). As a result of homology analysis, this amino acid sequence was found to show significant homology with proteins belonging to the immunoglobulin family. Among others, this amino acid sequence show high homology with A33 antigen that is a specific marker for human colon carcinoma [Proc. Natl. Acad. Sci. USA, 94, 469(1997)] and CAR (coxackie and adenovirus receptor) that is a virus receptor protein [Science, 275, 1320(1997)]. Judging from their primary structure, these factors are presumed to be a type I membrane protein. According to a hydrophilicity analysis on the basis of the amino acid sequence, 29 residues at the amino-terminus of A4RS-002 is estimated to be a secretion signal, and a sequence extending from the 249th to 270th amino acid is considered to be a highly hydrophobic transmembrane region. Since ICAM-1 and VCAM-1 belonging to the immunoglobulin family exhibit a shear stress-dependent alteration of expression, A4RS-002 is presumed to belong to the immunoglobulin family and function as a membrane protein. Its Northern blots exhibiting a shear stress-dependent increase of expression are shown in lane 76 of FIG. 2 and lane 21 of FIG. 4.

② A4RS-049

With respect to the full-length cDNA clone pfA4RS-049-1 obtained as a result of plaque hybridization, the whole nucleotide sequence of cDNA was determined and the resulting nucleotide sequence is shown as SEQ ID NO:145. Escherichia coli DH5 α strain having clone pfA4RS-049-1 introduced thereinto (Escherichia coli DH5 α /pfA4RS-049-1) was internationally deposited on August 5, 1999 with the National Institute of Bioscience and Human-Technology, the Agency of Industrial Science and Technology (Higashi 1-1-3, Tsukuba City, Ibaraki Prefecture, Japan) under the Budapest Treaty, and assigned the accession number FERM BP-6823. In the nucleotide sequence of A4RS-049, an ORF consisting of 881 amino acids was observed (its amino acid sequence is shown as SEQ ID NO:146). As a result of homology analysis, the protein encoded by A4RS-049 showed significant homology not only with mouse-derived 3BP-1 (SH3 domain binding protein)

[EMBO, J. 14, 3127(1995)], but also with various GTPase-activating protein (GAPs) such as rhoGAP and Abr. GAPs are a family of proteins controlling the GTPase activity of low-molecular-weight GTP-binding proteins such as ras and rab, and A4RS-049 shows homology with GAPs (e.g., rho and rac) specific for a subfamily considered to participate in regulation of cytoskeleton. In the amino acid sequence encoded by A4RS-049, the GTPase-activating domain conserved among known GAPs is present. Accordingly, A4RS-049 is presumed to function as a GAP. Moreover, a nematode-derived gene [Accession: Z73425] and a yeast-derived gene [Accession: Z97210], which are registered in databases but have an unknown function, show significant homology with the protein encoded by A4RS-049. Thus, A4RS-049 is expected to be a gene conserved well in the process of evolution. Its Northern blots exhibiting a shear stress-dependent increase of expression are shown in lane 77 of FIG. 2 and lane 22 of FIG. 4.

③ A4RS-230

With respect to the full-length cDNA clone pfA4RS-230-2 obtained as a result of plaque hybridization, the whole nucleotide sequence of cDNA was determined and the resulting nucleotide sequence is shown as SEQ ID NO:147. *Escherichia coli* DH5 α strain having clone pfA4RS-230-2 introduced thereinto (*Escherichia coli* DH5 α /pfA4RS-230-2) was internationally deposited on August 5, 1999 with the National Institute of Bioscience and Human-Technology, the Agency of Industrial Science and Technology (Higashi 1-1-3, Tsukuba City, Ibaraki Prefecture, Japan) under the Budapest Treaty, and assigned the accession number FERM BP-6824.

In the nucleotide sequence of A4RS-230, an ORF consisting of 322 amino acids was observed (its amino acid sequence is shown as SEQ ID NO:148). As a result of homology analysis, the protein encoded by A4RS-230 shows as high as 83% homology with mouse myeloid upregulated protein [Accession: 035682] and is considered to be a human counterpart thereof. However, its part on the C-terminal side is substantially different. As to mouse myeloid upregulated protein, only the sequence is registered in a database and its function is unknown. According to a hydrophilicity analysis on the basis of the amino acid sequence, the

protein encoded by A4RS-230 has very high hydrophobicity and may hence function as a membrane protein. However, a sequence judged to be a signal sequence is not present at the N-terminus. Its Northern blots exhibiting a shear stress-dependent increase of expression are shown in lane 78 of FIG. 2 and lane 23 of FIG. 4.

④ A4RS-239

With respect to the full-length cDNA clone pfA4RS-239-2 obtained as a result of plaque hybridization, the whole nucleotide sequence of cDNA was determined and the resulting nucleotide sequence is shown as SEQ ID NO:149. *Escherichia coli* DH5 α strain having clone pfA4RS-239-2 introduced thereinto (*Escherichia coli* DH5 α /pfA4RS-239-2) was internationally deposited on August 5, 1999 with the National Institute of Bioscience and Human-Technology, the Agency of Industrial Science and Technology (Higashi 1-1-3, Tsukuba City, Ibaraki Prefecture, Japan) under the Budapest Treaty, and assigned the accession number FERM BP-6825.

In the nucleotide sequence of A4RS-239, an ORF consisting of 663 amino acids was observed (its amino acid sequence is shown as SEQ ID NO:150). As a result of homology analysis, the protein encoded by A4RS-239 showed low but significant homology with various GAPs such as rhoGAP and Abr, similarly to the above-described A4RS-049. However, A4RS-239 and A4RS-049 are different DNAs. In the amino acid sequence encoded by A4RS-239, the GTPase-activating domain conserved among known GAPs is present. Accordingly, A4RS-239 is presumed to function as a GAP. Its Northern blots exhibiting a shear stress-dependent increase of expression are shown in lane 79 of FIG. 2 and lane 24 of FIG. 3.

⑤ A4RS-242

With respect to the full-length cDNA clone pfA4RS-242-1 obtained as a result of plaque hybridization, the whole nucleotide sequence of cDNA was determined and the resulting nucleotide sequence is shown as SEQ ID NO:151. *Escherichia coli* DH5 α strain having clone pfA4RS-242-1 introduced thereinto (*Escherichia coli* DH5 α /pfA4RS-242-1) was internationally deposited on August 5, 1999 with the National Institute of Bioscience and

Human-Technology, the Agency of Industrial Science and Technology (Higashi 1-1-3, Tsukuba City, Ibaraki Prefecture, Japan) under the Budapest Treaty, and assigned the accession number FERM BP-6826. In the nucleotide sequence of A4RS-242, an ORF consisting of 863 amino acids was observed (its amino acid sequence is shown as SEQ ID NO:152). As a result of homology analysis, the amino-terminal half of the protein encoded by A4RS-242 is identical with approximately the full length of the product of the gene *ehb10*. However, a part of A4RS-242 corresponding to the half on the carboxyl-terminal side is not present in *ehb10*. That is, they are considered to be splicing variants. *ehb10* is one of the proteins obtained by expression cloning, as factors binding to the EH domain considered to participate in the protein interaction of Eps15 (the substrate for EGF receptor) [Genes & Dev., 11, 2239(1997)], but its function is unknown. However, the motif required for binding to the EH domain is also present in A4RS-242. Its Northern blots exhibiting a shear stress-dependent increase of expression are shown in lane 80 of FIG. 2 and lane 25 of FIG. 4.

⑥ A4RS-491

With respect to the full-length cDNA clone pfA4RS-491-1 obtained as a result of plaque hybridization, the whole nucleotide sequence of cDNA was determined and the resulting nucleotide sequence is shown as SEQ ID NO:153. *Escherichia coli* DH5 α strain having clone pfA4RS-491-1 introduced thereinto (*Escherichia coli* DH5 α /pfA4RS-491-1) was internationally deposited on August 5, 1999 with the National Institute of Bioscience and Human-Technology, the Agency of Industrial Science and Technology (Higashi 1-1-3, Tsukuba City, Ibaraki Prefecture, Japan) under the Budapest Treaty, and assigned the accession number FERM BP-6827. In the nucleotide sequence of A4RS-491, an ORF consisting of 331 amino acids was observed (its amino acid sequence is shown as SEQ ID NO:154). As a result of homology analysis, the protein encoded by A4RS-491 was identical with an amino acid sequence [Accession: 043334] registered in a database as a human hypothetical protein over a wide range. However, this hypothetical protein consists of 393 amino acids, and it has been found that its 88th to 148th amino acids are not contained in the amino acid sequence encoded by A4RS-491. That is, they are considered to be splicing

variants. The protein encoded by A4RS-491 shows substantial homology with nematode-derived glycerophosphodiester phosphodiesterase [Accession: Z78198] and bacterium-derived glycerophosphodiester phosphodiesterase [Accession: E69827], and has been found to be a gene conserved well in the process of evolution. It is known that bacterium-derived glycerophosphodiester phosphodiesterase is present on membranes. According to a hydrophilicity analysis on the basis of the amino acid sequence encoded by A4RS-491, an amino acid sequence extending from the 1st to 26th amino acid in SEQ ID NO:154 is presumed to be a signal peptide. Its Northern blots exhibiting a shear stress-dependent increase of expression are shown in lane 81 of FIG. 2 and lane 26 of FIG. 4.

⑦ A4RS-578

With respect to the full-length cDNA clone pfA4RS-578-1 obtained as a result of plaque hybridization, the whole nucleotide sequence of cDNA was determined and the resulting nucleotide sequence is shown as SEQ ID NO:155. *Escherichia coli* DH5 α strain having clone pfA4RS-578-1 introduced thereinto (*Escherichia coli* DH5 α /pfA4RS-578-1) was internationally deposited on August 5, 1999 with the National Institute of Bioscience and Human-Technology, the Agency of Industrial Science and Technology (Higashi 1-1-3, Tsukuba City, Ibaraki Prefecture, Japan) under the Budapest Treaty, and assigned the accession number FERM BP-6828. In the nucleotide sequence of A4RS-578, an ORF consisting of 541 amino acids was observed (its amino acid sequence is shown as SEQ ID NO:156). As a result of homology analysis, the protein encoded by A4RS-578 showed the highest homology with the amino acid sequence [Accession: Z95559] of a protein of unknown function registered as a nematode-derived hypothetical protein, and then showed significant homology with rat brain finger protein (BFP) [Biochem. Biophys. Res. Commun., 240, 8(1997)]. Rat BFP was cloned as a novel gene having the RING finger motif as a kind of zinc finger motif, and it has been reported that this gene is expressed brain-specifically and that the expression of this gene may be induced at the stage of differentiation into nerve cells. However, a sequence judged to be the RING finger motif is not present in the amino acid sequence encoded by A4RS-578. The protein encoded by A4RS-578 also shows significant

homology with various GTP-binding proteins, and has two of the three motifs possessed in common by many GTP-binding proteins. Since the existence of GTP-binding proteins having only two motifs has been reported, there is a possibility that the protein encoded by A4RS-578 functions as a GTP-binding protein. Its Northern blots exhibiting a shear stress-dependent increase of expression are shown in lane 82 of FIG. 2 and lane 27 of FIG. 4.

⑧ A4RS-829

With respect to the full-length cDNA clone pfA4RS-829-1 obtained as a result of plaque hybridization, the whole nucleotide sequence of cDNA was determined and the resulting nucleotide sequence is shown as SEQ ID NO:157. *Escherichia coli* DH5 α strain having clone pfA4RS-829-1 introduced thereinto (*Escherichia coli* DH5 α /pfA4RS-829-1) was internationally deposited on August 5, 1999 with the National Institute of Bioscience and Human-Technology, the Agency of Industrial Science and Technology (Higashi 1-1-3, Tsukuba City, Ibaraki Prefecture, Japan) under the Budapest Treaty, and assigned the accession number FERM BP-6829. In the nucleotide sequence of A4RS-829, an ORF consisting of 173 amino acids was observed (its amino acid sequence is shown as SEQ ID NO:158). As a result of homology analysis, the protein encoded by A4RS-829 showed substantial homology with the amino acid sequences of proteins of unknown functions registered as hypothetical proteins, such as an arabidopsis-derived protein [Accession: 048707], a nematode-derived protein [Accession: Q20340] and a yeast-derived protein [Accession: Q03677], and was found to be a gene conserved well in the process of evolution. Its Northern blots exhibiting a shear stress-dependent increase of expression are shown in lane 83 of FIG. 2 and lane 28 of FIG. 4.

Example 6

Production of a recombinant protein of A4RS-002

(1) Construction of an expression plasmid

To 2 μ g of pfA4RS-002-1 obtained in Example 5, 5 μ l of 10-reaction buffer (attached to the enzyme), 1 μ l of *Xho*I (10 units/ μ l; manufactured by Takara Shuzo Co., Ltd.), and distilled water were added so as to make a total volume of 50 μ l. This mixture incubated at

37°C for 2 hours to digest the cDNA completely. Then, 0.5 µl of 5 M NaCl and 1 µl of NotI (10 units/µl; manufactured by Takara Shuzo Co., Ltd.) were added to the reaction mixture. This mixture incubated at 37°C for 2 hours to digest the cDNA completely. The reaction mixture was subjected to a phenol-chloroform treatment and a chloroform treatment, and then precipitated with ethanol. After the resulting precipitate was dissolved in 20 µl of distilled water, 3 µl of 10 x blunting buffer (attached to the enzyme), 6 µl of a 2.5 mM mixed dNTP solution, and 1 µl of Klenow fragment (manufactured by Takara Shuzo Co., Ltd.) were added thereto. This mixture was incubated at 37°C for 1 hour to carry out the blunting of restriction enzyme-treated ends. The reaction mixture was subjected to a phenol-chloroform treatment and a chloroform treatment, and then precipitated with ethanol. After the resulting precipitate was dissolved in 5 µl of distilled water, 0.4 µg and 0.3 µg, respectively, of SfiI linkers (5'-CTTTAGAGCAC-3', 5'-CTCTAAAG-3') were added thereto so as to make a volume of 6 µl. Twelve µl of solution I and 6 µl of solution II of a Ligation Kit Ver. 2 (manufactured by Takara Shuzo Co., Ltd.) were added thereto, and the resulting mixture was incubated at 16°C overnight to carry out linker ligation. The total amount of the reaction mixture was electrophoresed through 0.8% agarose gel, and the desired fragments were recovered using a QIAEX II Gel Extraction Kit (manufactured by QIAGEN). The procedure therefor was carried out according to the manual attached to the kit. The recovered DNA fragments were dissolved in 10 µl of distilled water. To this insert DNA, an animal cell expression plasmid vector pAMo [J. Biol. Chem., 268, 22782(1993); also called pAMoPRC3Sc (Japanese Published Unexamined Patent Application No. 336963/93)], which had been linearized with SfiI and similarly recovered from an agarose gel, was added in a molar amount equal to 1/5 of that of the insert, and Ligation High (manufactured by Toyobo Co., Ltd.) in a volume equal to that of the solution. This mixture was incubated at 16°C for 3 hours to ligate the insert with linkers to the vector, and then introduced into competent-cell Escherichia coli MW294. After introduction, LB agar medium containing 50 µg/ml ampicillin was inoculated with the bacterial suspension and incubated at 37°C overnight to form colonies. The resulting colonies were randomly picked up, and the plasmid was obtained from each colony and examined for

the presence or absence of the insert by a restriction enzyme treatment. With respect to the colonies having the insert, the direction of the insert was examined. Using one clone, pAMo-002, having the desired directivity, the plasmid was mass-prepared using a QIAGEN Plasmid Midi Kit (manufactured by QIAGEN). The procedure therefor was carried out according to the manual attached to the kit. This plasmid was sterilely precipitated with ethanol and then dissolved in distilled water to a concentration of 1 µg/µl. The above-described construction of pAMo-002 is illustrated in FIG. 5.

(2) Introduction of the recombinant plasmid into cultured animal cells

Namalwa KJM-1 [Cytotechnology, 1, 151(1988)], which is a host cell for gene expression, was collected by centrifugation, washed with 10 ml of K-PBS [13.7 mM KCl, 0.27 mM NaCl, 0.81 mM Na₂HPO₄, 0.15 mM KH₂PO₄, 0.4 mM MgCl₂], and suspended in cooled K-PBS so as to have a density of 8×10^6 cells/ml. Two hundred µl (1.6×10^6 cells) of this cell suspension was mixed with 4 µl (4 µg) of the plasmid DNA prepared in step (1), and this mixture was quickly transferred to a chamber (manufactured by BIO-RAD) which had previously been cooled on ice. Then, using a Gene Pulser (manufactured by BIO-RAD), electroporation was carried out by the application of a voltage of 0.35 kV, 125 µF. Thereafter, the chamber was quickly placed on ice, and the electroporated cells were transferred to a flask containing 8 ml of RPMI1640 medium (manufactured by Nissui Seiyaku Co., Ltd.). After the flask was incubated for 24 hours under conditions include 37°C and 5% CO₂, G-418 as an agent for selection was added thereto so as to give a final concentration of 0.5 mg/ml. Gene-introduced cells were selected by continuing the incubation for one more week. As a control, KJM-1 cells into which only pAMo vector having no insert was introduced were also prepared.

Example 7

Cloning of full-length cDNAs (2)

Similarly to Example 5, with respect to three novel partial cDNA fragments obtained from the subtraction library, full-length cDNAs were obtained from full-length cDNA libraries derived from human adipose tissue or Kato III.

(1) Construction of full-length cDNA libraries derived from human adipose tissue or KatoIII cells

mRNA was extracted from human adipose tissue according to the method described in the paper (J. Sambrook, E.F. Fritsch & T. Maniatis, *Molecular Cloning*, Second Edition, Cold Spring Harbor Laboratory Press, 1989). Moreover, poly(A)⁺ RNA was purified with oligo-dT cellulose.

Similarly, mRNA was extracted from KatoIII cells according to the method described in the paper (J. Sambrook, E.F. Fritsch & T. Maniatis, *Molecular Cloning*, Second Edition, Cold Spring Harbor Laboratory Press, 1989). Moreover, poly(A)⁺ RNA was purified with oligo-dT cellulose.

From each poly(A)⁺ RNA, a cDNA library was constructed according to an oligo-cap method [M. Maruyama and S. Sugano, *Gene*, 138, 171-174(1994)]. Using an oligo-cap linker (SEQ ID NO:162) and an oligo-dT primer (SEQ ID NO:163), BAP (Bacterial Alkaline Phosphatase) treatment, TAP (Tabacco Acid Phosphatase) treatment, RNA ligation, the synthesis of first-strand cDNA, and the removal of RNA were carried out according to the methods described in the paper [Suzuki & Kanno, *Tanpakushitsu-Kakusan-Koso* (in Japanese), 41:197-201(1996); Y. Suzuki, *Gene*, 200, 149-156(1997)]. Then, the resulting cDNA was converted to double-stranded cDNA by PCR using two primers [a 5'-terminal sense primer (SEQ ID NO:164) and a 3'-terminal antisense primer (SEQ ID NO:165)], and then cleaved with SfiI. This PCR was carried out in such a way that, using a commercially available GeneAmp XL PCR Kit (manufactured by Perkin Elmer), the reaction mixture was heat-treated at 95°C for 5 minutes, subjected 12 times to a reaction cycle comprising heating at 95°C for 1 minute, 58°C for 1 minute, and at 72°C for 10 minutes, and then kept at 4°C. Thereafter, a cDNA library was constructed by cloning the cDNA into a DraIII-cleaved pME18SFL3 vector [Accession: AB009864; expression vector, 3392 bp] with the fixed directivity of the cDNA.

(2) Determination of the full-length cDNA sequences

With respect to the plasmid DNAs of clones obtained from the cDNA libraries prepared in step (1), each cDNA clone was subjected to an in vitro transposon (hereinafter abbreviated as Tn) transposition reaction by using a GSP-1 Genome Priming System (manufactured by NEB). pGPS1.1 (manufactured by NEB) was used as the Tn donor. After completion of the Tn transposition reaction, a portion of the DNA sample was taken and used to transform Escherichia coli. Typically, 16 Tn-inserted clones were picked up for each cDNA clone. With respect to the plasmid DNAs of clones thus obtained, the full-length cDNA sequences were determined in the same manner as in Example 5, by using Primer N (SEQ ID NO:166) and Primer S (SEQ ID NO:167) as primers.

(3) Novel full-length genes exhibiting a shear stress-dependent increase of expression

Using the sequence of A4RS-011 obtained from the subtraction library in Example 3 as a query, the cDNA sequences obtained in step (2) were searched by means of BLAST program [Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schaffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman, *Nucleic Acids Res.*, 25, 3389-3402(1997)]. As a result, the sequence of A4RS-011 was identical with C-KAT07969 (SEQ ID NO:168). Among the ORFs of this cDNA sequence, the longest translated amino acid sequence was regarded as the amino acid sequence (121-1062; SEQ ID NO:169) encoded by the cDNA sequence of C-KAT07969. This amino acid sequence does not show substantial homology with other known proteins. Its Northern blot exhibiting a shear stress-dependent increase of expression is shown in panel 56 of FIG. 2.

Using the sequence of A4RS-604 obtained from the subtraction library in Example 3 as a query, the cDNA sequences obtained in step (2) were searched by means of BLAST program. As a result, the sequence of A4RS-604 was identical with the sequence of C-ADKA02341 (SEQ ID NO:170). This sequence is identical with a portion of the sequence of *H. sapiens* mRNA for myosin-I beta [Accession: X98507]. Among the ORFs of this cDNA sequence, the longest translated amino acid sequence was regarded as the amino acid sequence (SEQ ID NO:171) encoded by the cDNA sequence of C-ADKA02341. Its Northern

blots exhibiting a shear stress-dependent increase of expression are shown in panel 64 of FIG. 2 and panel 18 of FIG. 4.

Using the sequence of A4RS-619 obtained from the subtraction library in Example 3 as a query, the cDNA sequences obtained in step (2) were searched by means of BLAST program. As a result, the sequence of A4RS-619 was identical with the sequence of C-hep01279 (SEQ ID NO:172). Among the ORFs of this cDNA sequence, the longest translated amino acid sequence was regarded as the amino acid sequence (SEQ ID NO:173) encoded by the cDNA sequence of C-hep01279. This amino acid sequence does not show substantial homology with other known proteins. Its Northern blot exhibiting a shear stress-dependent increase of expression is shown in panel 66 of FIG. 2.

Example 8

Detection of the apoptosis-suppressing activity of A4RS-041

In order to investigate the function of genes exhibiting a shear stress-dependent increase of expression which were obtained from the subtraction library, the following experiments were carried out with respect to a gene of unknown function, A4RS-041, having homology with the gene LFG capable of suppressing Fas-mediated apoptosis.

(1) Construction of a recombinant virus vector

Using a plasmid having the full-length A4RS-041 (SEQ ID NO:7) as a template, the part of the cDNA sequence which encodes the protein of A4RS-041 was specifically amplified by PCR. That is, in a PCR tube, 20 ng of the template plasmid DNA, 25 pmol of a 5'-terminal sense primer having the HindIII site added thereto (SEQ ID NO: 174), 25 pmol of a 3'-terminal antisense primer having the ClaI site added thereto (SEQ ID NO: 175), 5 μ l of 10 x reaction buffer (attached to the enzyme), 5 μ l of a 2 mM dNTP solution, and 0.5 μ l of KOD DNA polymerase (2.5 units/ μ l; manufactured by Toyobo Co, Ltd.) were mixed, and sterilized water was added thereto so as to make a volume of 50 μ l. This mixture was heated at 98°C for 15 seconds, at 65°C for 2 seconds, and at 74°C for 30 seconds. This cycle was repeated 25 times to amplify the cDNA. The resulting amplified fragment of the full-length A4RS-041 was purified by cleaving its ends with HindIII and ClaI, and ligated to virus vector

pCLNCX (manufactured by IMGENEX) which had previously been cleaved with HindIII and ClaI. As a result, there was constructed a recombinant virus vector pCLNC041 with which the expression of A4RS-041 is induced by CMV promoter. With respect to the resulting recombinant virus vector pCLNC041, the nucleotide sequence of its inserted fragment part was determined. Thus, it was confirmed that no nucleotide substitution was caused by PCR. As a control, pCLNCGFP was constructed by inserting EFGP (enhanced green fluorescent protein; manufactured by Clontech) into the HindIII and ClaI sites of pCLNCX in the same manner.

(2) Preparation of HeLa cells expressing A4RS-041 to be highly and stably

Recombinant viruses were produced by introducing each of the recombinant virus vectors constructed in step (1) into 293 cells for virus production. For the transfection of 293 cells with pCLNC041 or pCLNCGFP, a TransFast (manufactured by Promega) was used. The procedure therefor was carried out according to the attached manual. The procedures for virus production and for the infection of HeLa cells were carried out according to the manual attached to the virus vector (manufactured by IMGENEX) used.

Two days after infection, 300 µg/ml of G418 (manufactured by life Technologies) was added to the HeLa cells and the incubation was continued, so that uninfected cells were selectively eliminated. According to this procedure, there were obtained transformants expressing A4RS-041 or GFP to be highly and stably.

(3) Detection of an apoptosis-suppressing activity

Apoptosis was induced by adding 100 ng/ml of anti-Fas monoclonal antibody CH-11 (manufactured by MBL) to the stable transformants of HeLa cells obtained in step (2) (i.e., the stable transformants of HeLa cells expressing the expression of A4RS-041 or GFP as a control). Twenty-four (24), 36 and 48 hours after the start of induction, the survival rate of the cells was measured by staining with trypan blue. For this purpose, the survival rate was measured for both suspended cells and adhering cells. All experiments were carried out in duplicate, and averages and standard deviations were obtained. The results are shown in FIG. 6A. Moreover, when the antibody concentration was altered to 10, 50, 100 and 500 ng/ml,

the survival rate after 36 hours was measured. The results are shown in FIG. 6B. In the HeLa cells having A4RS-041 introduced thereinto (represented by □ in FIG. 6), a significant increase in survival rate was observed at all points as compared with the HeLa cells having GFP (control) introduced thereinto (represented by ■ in FIG. 6). Thus, it has been found that, at least in HeLa cells, A4RS-041 has an activity for suppressing Fas-mediated apoptosis.

Example 9

Analysis of the distribution of expression of A4RS-041

With respect to A4RS-041 that was found to have an apoptosis-suppressing activity in Example 8, the following experiments were carried out in order to examine its sites of expression in human tissues.

(1) Analysis of the expression of A4RS-041 in human normal tissues

Using primers specific for A4RS-041 (SEQ ID NO:176,177) and a PCR DIG Labeling Mix (manufactured by Boehringer Mannheim), a template comprising the A4RS-041-containing plasmid obtained in Example 2 was subjected to PCR. Thus, a DIG-labeled A4RS-041 specific fragment was prepared. Using this DNA fragment as a probe, hybridization was carried out with a Human Multiple Tissue Northern Blot (manufactured by Clontech) on which RNAs derived from 8 human tissues had been blotted. After washing, chemiluminescence signals were detected using a DIG luminescence detection kit (manufactured by Boehringer Mannheim). The procedure therefor was carried out according to the manual attached to the kit. As shown in panel A of FIG. 7, signals specific for A4RS-041 were detected in the vicinity of about 2.5 kb. In Lanes 1 to 8, 2 µg each of poly(A)⁺ RNAs derived from spleen, kidney, skeletal muscle, liver, lung, placenta, brain and heart had been electrophoresed. Although signals were observed in all lanes, the signal in lane 7 (brain) was weak. Thus, it has been found that the expression of A4RS-041 is relatively low in the brain. On the other hand, it has been reported that the expression of LFG is very high in the brain and low in the periphery [Proc. Natl. Acad. Sci. USA, 22, 12673-12678(1999)]. This suggests that A4RS-041 and LFG function tissue-specifically.

(2) Investigation on the expression of A4RS-041 and LFG in human vascular endothelial cells and brain

Using a template comprising 1 µg of the HUVEC (having no shear stress applied thereto)-derived poly(A)⁺ RNA obtained in Example 2 or 1 µg of human brain-derived poly(A)⁺ RNA (manufactured by Clontech), single-stranded cDNA was synthesized using a Superscript Preamplification System (manufactured by Life Technologies). The procedure therefor was carried out according to the manual attached to the kit. The finally obtained cDNA solution was diluted to 5 ml and used for PCR. Using these cDNAs as templates, PCR was carried out using primers specific for A4RS-041 (SEQ ID NO:176,177), LFG (SEQ ID NO:178,179) and G3PDH (SEQ ID NO:180,181). The reaction mixture contained 5 µl of a cDNA solution, 2 µl of 10 x reaction buffer (attached to the enzyme), 1.6 µl of a 2.5 mM dNTP solution, 1 µl of dimethyl sulfoxide, 10 pmol each of sense and antisense primers, and 0.1 µl of GeneTaq DNA polymerase (5 units/µl; manufactured by Nippon Gene Co., Ltd.), and sterilized water was added thereto so as to make a total volume of 20 µl. After the template and the primers were denatured by heating at 94°C for 1 minute, a cycle comprising heating at 94°C for 1 minute, at 60°C for 1 minute, and at 72°C for 1 minute was repeated. The number of cycles was 33 for A4RS-041 and LFG, and 24 for G3PDH. The reaction mixture was kept at 72°C for 10 minutes and then cooled to 4°C. One-half of the resulting PCR product was subjected to 1.8% agarose electrophoresis. The results are shown in panel B of FIG. 7. In lane 1, a 100 bp ladder (manufactured by Life Technologies) was electrophoresed as a size marker. Lanes 2, 4 and 6 show the PCR products obtained with HUVEC-derived cDNA, and Lanes 3, 5 and 7 show the PCR products obtained with human brain-derived cDNA. Moreover, lanes 2 and 3 show the PCR products obtained with A4RS-041-specific primers, lanes 4 and 5 show the PCR products obtained with LFG-specific primers, and lanes 6 and 7 show the PCR products obtained with G3PDH-specific primers.

The band of A4RS-041 is amplified in both HUVECs (lane 2) and the brain (lane 3), indicating that A4RS-041 is expressed in both of them. Its expression level in the brain tends to be lower than in HUVECs. On the other hand, LFG is very strongly expressed in the

brain(lane 5), but the band of LFG is not amplified at all in HUVECs (lane 4), and this indicates that LFG is not expressed in HUVECs.

From the above-described results, it is believed that the factor involved in the suppression of apoptosis in endothelial cells is not LFG but A4RS-041.

The homology between the amino acid sequences of A4RS-041 and LFG (human-derived) is shown in FIG. 8. They are judged to be homologous proteins having 48.9% (152/311) identity. However, it has been found that a portion corresponding to about one-third on the N-terminal side has considerably low homology.

FREE TEXT FOR SEQUENCE LISTING

SEQ ID NO:159 - Description of artificial sequence: Artificial synthetic primer sequence

SEQ ID NO:160 - Description of artificial sequence: Artificial synthetic primer sequence

SEQ ID NO:161 - Description of artificial sequence: Artificial synthetic primer sequence

SEQ ID NO:162 - Description of artificial sequence: Oligo-cap linker sequence

SEQ ID NO:163 - Description of artificial sequence: Oligo-dT primer sequence

SEQ ID NO:164 - Description of artificial sequence: Artificial synthetic primer sequence

SEQ ID NO:165 - Description of artificial sequence: Artificial synthetic primer sequence

SEQ ID NO:166 - Description of artificial sequence: Artificial synthetic primer sequence

SEQ ID NO:167 - Description of artificial sequence: Artificial synthetic primer sequence

SEQ ID NO:174 - Description of artificial sequence: Synthetic DNA

SEQ ID NO:175 - Description of artificial sequence: Synthetic DNA

SEQ ID NO:176 - Description of artificial sequence: Synthetic DNA

SEQ ID NO:177 - Description of artificial sequence: Synthetic DNA

SEQ ID NO:178 - Description of artificial sequence: Synthetic DNA

SEQ ID NO:179 - Description of artificial sequence: Synthetic DNA

SEQ ID NO:180 - Description of artificial sequence: Synthetic DNA

SEQ ID NO:181 - Description of artificial sequence: Synthetic DNA